



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/58, 33/533, C12N 15/65, C07K 14/435, G01N 33/542</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/48278</b> <b>(43) International Publication Date:</b> 29 October 1998 (29.10.98)
<b>(21) International Application Number:</b> PCT/US98/08109 <b>(22) International Filing Date:</b> 23 April 1998 (23.04.98) <b>(30) Priority Data:</b> 08/842,322      24 April 1997 (24.04.97)      US <b>(71) Applicant:</b> UNIVERSITY OF ROCHESTER [US/US]; Office of Technology Transfer, 518 Hylan Building, Rochester, NY 14627 (US). <b>(72) Inventor:</b> PERSECHINI, Anthony; 6 Sunnybrook Lane, Fairport, NY 14450 (US). <b>(74) Agents:</b> GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> DETECTION OF INTRACELLULAR LIGAND BINDING		
<b>(57) Abstract</b>  A method of monitoring the amount of primary or secondary ligand in a cell, using a green fluorescent protein complex and fluorescence resonance energy transfer between two GFP molecules to monitor the primary or secondary ligand, is disclosed. Further disclosed is a method of screening a molecule, such as a peptide, for primary ligand-binding activity, which also uses a green fluorescent protein complex and fluorescence resonance energy transfer between two GFP molecules, to screen the molecule or peptide. A green fluorescent protein complex comprising a first green fluorescent protein, a primary ligand-binding peptide, and a second green fluorescent protein, is also disclosed.		

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## DETECTION OF INTRACELLULAR LIGAND BINDING

5           The subject matter of this application was made with support from the United States Government under grant no. DK44322-03 of the National Institutes of Health.

### FIELD OF THE INVENTION

10           The present invention relates generally to cell monitoring and ligand detection and evaluation, and more particularly to a method of monitoring the level of a primary ligand in a cell or of evaluating a molecule for primary ligand-binding activity, using a green fluorescent protein fusion complex that exhibits ligand-dependent fluorescence resonance energy transfer.

### BACKGROUND OF THE INVENTION

15           Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

20           The activity of a cell is influenced and regulated by many molecules. Many cells have receptors which activate pathways within the cell, such as the cAMP pathway or the phosphoinositide pathway. Such receptors are often activated by a ligand. When the ligand binds to its receptor, the receptor activates the pathway. Other molecules influential in a cell include ions such as calcium ions. In an attempt to understand the

25           molecular biology of a cell, the ways in which cells interact, or even causes of cell death, more knowledge is continually needed regarding the distribution and/or pattern of expression of various molecules within a cell or within a tissue or organ.

          One example of such a molecule is calmodulin. Calmodulin modulates the activities of a large and constantly increasing number of known target proteins (Bredt and Snyder 1990; Cho et al. 1992; Edelman et al. 1996; Crivici and Ikura 1995; Kink et al. 1990; Lu and Means 1993; Manalan and Klee 1984; Means et al. 1991; Wang et al. 1996). Through their actions it participates in the regulation of most processes in the cell including motility, axonal transport, synaptic transmission, ion homeostasis, cell cycle progression, gene expression and apoptosis. It is of interest to determine whether

maximal and resting free  $(Ca^{2+})_4$ -calmodulin levels vary among cell types, or at different points in the cell cycle, which has been shown to be associated with cyclic changes in total calmodulin levels. It would also be of interest to investigate spatio-temporal changes in calmodulin activity during cellular events that involve it.

5 It would also be of interest to determine maximal and resting levels of other molecules and ligands, in various cell types, or at different points in the cell cycle, and well as to determine spatio-temporal changes in ligand-binding activity during cellular events that involve it.

## 10 SUMMARY OF INVENTION

To this end, the subject invention provides a method of monitoring the amount of a primary or secondary ligand in a cell. The method first involves the construction of a green fluorescent protein (GFP) complex. The green fluorescent protein complex comprises a first green fluorescent protein which is excited at a first wavelength and  
15 which emits fluorescence at a second wavelength; a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal  
20 end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. The constructed green fluorescent protein complex is then introduced into a cell, and a base amount of fluorescence emission at the third wavelength when the cell is excited at the first wavelength is determined. This determination provides a control or base amount of fluorescence resonance energy  
25 transfer between the two green fluorescent proteins of the green fluorescent protein complex. The amount of fluorescence emission over time at the third wavelength when the cell is excited over time at the first wavelength is determined. Next, a comparison is made of the amount of fluorescence emission over time to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base  
30 amount of fluorescence emission indicates an increase in the amount of the primary ligand in the cell. In particular, the primary ligand present in the cell has bound to the primary ligand-binding peptide, changing the distance between the two GFP molecules and altering fluorescence resonance energy transfer (FRET) between them. Changes in



the amount of fluorescence emission over time can be used to monitor changes in the amount of the primary ligand in the cell over time.

By quantitating the amount of fluorescence emission over time using the subject method, one can estimate the amount of the primary ligand in the cell over time.

5       The subject invention further provides a method for monitoring the amount of a secondary ligand in the cell, wherein the secondary ligand binds to the primary ligand and is necessary for binding of the primary ligand to the primary ligand-binding peptide. This method is as recited above, except the method further comprises introducing a known concentration of the primary ligand into the cell prior to determining the base  
10       amount of fluorescence emission. When the amount of fluorescence emission is less than the base amount of fluorescence emission, it is an indication of the presence of the secondary ligand. As above, changes in the amount of fluorescence emission over time indicate changes in the amount of the secondary ligand in the cell over time.

      The invention further provides a method of screening a peptide for primary  
15       ligand-binding activity. The method comprises constructing a green fluorescent protein complex. In this embodiment, the green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein as described above, but they are covalently attached by a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to  
20       the first green fluorescent protein and the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell in the absence of the primary ligand, and a determination of the base amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. The candidate primary ligand is  
25       then added to the cell, and a determination of the amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the molecule has primary ligand-binding activity.  
30       Alternatively, this method of screening a peptide for primary ligand-binding activity can be performed in vitro in, for example, a test tube or well. The green fluorescent protein complex is expressed in a cell and then purified from the cell by conventional protein purification techniques (such as affinity chromatography). The purified protein complex

is placed in a test tube or well and a determination is made of the base amount of fluorescence emission at the third wavelength when the purified protein complex is excited at the first wavelength. The candidate primary ligand is then added to the test tube or well, and a determination is made of the amount of fluorescence emission at the third wavelength when the purified protein complex (in the presence of purified primary ligand) is excited at the first wavelength. As in the previous embodiment, a comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the peptide has primary ligand-binding activity.

Further provided is another method of screening a molecule for the ability to bind a primary ligand in competition with the primary ligand-binding peptide where the constructed green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein covalently attached by a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end. The amino terminal end of the primary ligand-binding peptide is covalently attached to the first green fluorescent protein and the carboxy terminal end of the primary ligand-binding peptide is covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell or test vesicle (such as a test tube or test well) with the primary ligand, and a determination is made of the base amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle are excited at the first wavelength. A molecule, such as a peptide, is then added to the cell or test vesicle, and a determination is made of the amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission greater than the base amount of fluorescence emission indicates that the molecule has primary ligand-binding activity. In particular, the molecule has bound the primary ligand thereby preventing the primary ligand from interfering with energy transfer between the two GFPs (the primary ligand does this by binding to the primary ligand-binding peptide that covalently attaches the two GFPs).

The invention further provides a green fluorescent protein complex which comprises a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide

which reversibly binds a primary ligand, the primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at the second wavelength and  
5 which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. When the green fluorescent protein complex is excited at the first wavelength, the amount of fluorescence emission at the third wavelength is reduced when the primary ligand-binding peptide is bound to primary ligand as compared to the amount of  
10 fluorescence emission at the third wavelength when the primary ligand-binding peptide is not bound to the primary ligand. Also provided is a cell which expresses the green fluorescent protein complex.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 illustrates the conformational change undergone by FIP-CB<sub>SM</sub> upon binding (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin;

20 Fig. 2 illustrates the calmodulin-dependent changes in the FIP-CB<sub>SM</sub> fluorescence emission spectrum;

Fig. 3 characterizes the calmodulin binding by FIP-CB<sub>SM</sub>, showing the titration of a mixture of 8.2 nM FIP-CB<sub>SM</sub> and 200 nM calmodulin with increasing concentrations of free Ca<sup>2+</sup> ion;

25 Fig. 4 characterizes the calmodulin binding by FIP-CB<sub>SM</sub>, showing the binding of (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin to FIP-CB<sub>SM</sub> at concentrations of 1 (■) and 1.4 (▲) nM;

Fig. 5 shows the emission spectra for titration of FIP-CB<sub>SM</sub> at a 1.4 nM concentration;

30 Figs. 6-11 show time courses for area-normalized enhanced fluorescence in cells microinjected with FIP-CB<sub>SM</sub>;

Fig. 12 illustrates the conformational change undergone by FIP-CA<sub>3</sub> upon binding Ca<sup>2+</sup>;

Fig. 13 shows a time course for area-normalized enhanced fluorescence in cells microinjected with FIP-CA<sub>3</sub>; and

Fig. 14 characterizes the calcium binding by FIP-CA<sub>3</sub>, showing the titration of FIP-CA<sub>3</sub> with increasing concentrations of free Ca<sup>2+</sup> ion.

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### DETAILED DESCRIPTION

The subject invention provides a method of monitoring the amount of a primary or secondary ligand in a cell. The method first involves the construction of a green fluorescent protein complex. The green fluorescent protein complex comprises a first  
10 green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at the second wavelength and  
15 which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. The constructed green fluorescent protein complex is then introduced into a cell and a base amount of fluorescence emission at the third wavelength when the cell is excited at the first wavelength is determined. This determination provides a control or  
20 base amount of fluorescence resonance energy transfer between the two green fluorescent proteins of the green fluorescent protein complex. The amount of fluorescence emission over time at the third wavelength when the cell is excited over time at the first wavelength is determined. Next, a comparison is made of the amount of fluorescence emission over time to the base amount of fluorescence emission, wherein  
25 an amount of fluorescence emission less than the base amount of fluorescence emission indicates an increase in the amount of the primary ligand in the cell. Changes in the amount of fluorescence emission over time can be used to monitor changes in the amount of the primary ligand in the cell over time.

By quantitating the amount of fluorescence emission over time using the subject  
30 method, one can estimate the amount of the primary ligand in the cell over time.

As used herein, a primary ligand refers to any molecule binding directly to the primary ligand-binding peptide in the GFP complex. An example of a primary ligand in accordance with the subject invention is calmodulin (where the primary ligand-binding

peptide is a calmodulin-binding peptide). Various calmodulin-binding peptides are known in the art and each can be used to tether the two green fluorescent proteins together. One calmodulin-binding peptide has the amino acid sequence as shown in SEQ ID NO:1. Examples of additional calmodulin-binding peptides are shown in Table 1 (see Crivici and Ikura 1995, and Persechini et al. 1996).

As further used herein, a green fluorescent protein refers to any fluorescent protein containing a naturally occurring fluorophore. The only requirement is that the two selected green fluorescent proteins have different excitation and fluorescence emission wavelengths. This is necessary to evaluate the fluorescence resonance energy transfer between the two proteins. Two examples of green fluorescent proteins which can be used in accordance with the subject invention are the red-shifted green fluorescent protein (which is excited at a wavelength maxima of 495 nm and emits fluorescence at a wavelength maxima of 505 nm) and the blue-shifted green fluorescent protein (which is excited at a wavelength maxima of 380 nm and emits fluorescence at a wavelength maxima of 440 nm). Other potentially suitable green fluorescent proteins include the GFP from *Aequorea victoria* (U.S. Patent No. 5,491,084;). A plasmid encoding the GFP of *Aequorea victoria* is available from the ATCC as Accession No. 75547. A mutated form of this GFP (a red-shifted mutant form) designated pRSGFP-C1 is commercially available from Clontech Laboratories, Inc. (Palo Alto, California). For further discussions of various GFP molecules, see Crameri et al. 1996; Delegrave et al. 1995; Gura 1996; Inouye and Tsuji 1994; Marshall et al. 1995; Ormo et al. 1996; Yang et al. 1996; and Youvan and Michel-Beyerle 1996.

In the case of the primary ligand calmodulin, as with any other appropriate primary ligand, the binding of calmodulin by the calmodulin-binding peptide may be dependent on the presence of a secondary ligand which binds to the primary ligand. For example, calmodulin requires the presence of calcium ion in order to bind to the calmodulin-binding peptide having SEQ ID NO:1. In these cases, the method of the subject invention further comprises introducing into the cell the secondary ligand necessary for binding of the primary ligand to the primary ligand-binding peptide. The amount of the secondary ligand in the cell can thereby be monitored by introducing the primary ligand into the cell prior to determining the base amount of fluorescence emission. When the amount of fluorescence emission is less than the base amount of fluorescence emission, it is an indication of the presence of the secondary ligand in the

cell. Furthermore, changes in the amount of fluorescence emission over time indicate changes in the amount of the secondary ligand in the cell over time. One embodiment of the subject invention thus provides for the monitoring of such secondary ligands within cells. This is very advantageous, especially for the monitoring of secondary ligands such as the calcium ion, because the level of calcium ion in a cell can be quantitated by indirect detection via the calcium ion's interaction with calmodulin and the calmodulin-binding peptide. In this embodiment, the primary ligand can be introduced into the cell by covalently attaching the primary ligand to one of the first green fluorescent protein or the second green fluorescent protein in the green fluorescent protein complex. This fixes the concentration of the primary ligand in relation to the indicator.

Other examples of secondary ligands in accordance with the subject invention include metal ions.

As used herein, covalent attachment refers to a non-reversible attachment whereas "bound" or "binding" refer to reversible attachments.

The invention further provides a method of screening a peptide for primary ligand-binding activity. The method comprises constructing a green fluorescent protein complex. In this embodiment, the green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein as described above, but they are covalently attached by a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to the first green fluorescent protein and the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell in the absence of the primary ligand, and a determination of the base amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. The primary ligand is then added to the cell, and a determination of the amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the peptide has primary ligand-binding activity. Preferably, the primary ligand is calmodulin. Alternatively, this method of screening a peptide for primary ligand-binding activity can be performed in vitro in, for example, a

test tube or well. The green fluorescent protein complex is expressed in a cell and then purified from the cell by conventional protein purification techniques (such as affinity chromatography). The purified protein complex is placed in a test tube or well and a determination is made of the base amount of fluorescence emission at the third wavelength when the purified protein complex is excited at the first wavelength. Purified primary ligand is then added to the test tube or well, and a determination is made of the amount of fluorescence emission at the third wavelength when the purified protein complex (in the presence of purified primary ligand) is excited at the first wavelength. As in the previous embodiment, a comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the peptide has primary ligand-binding activity.

Further provided is a method of screening a molecule for the ability to bind a known primary ligand where the constructed green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein covalently attached by a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end. The amino terminal end of the primary ligand-binding peptide is covalently attached to the first green fluorescent protein and the carboxy terminal end of the primary ligand-binding peptide is covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell or test vesicle with the primary ligand, and a determination is made of the base amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle is excited at the first wavelength. A molecule, such as a peptide, is then added to the cell or test vesicle, and a determination is made of the amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission greater than the base amount of fluorescence emission indicates that the molecule has primary ligand-binding activity. As above, preferably the primary ligand is calmodulin and the primary ligand-binding peptide (a calmodulin-binding peptide) has an amino acid sequence as shown in SEQ ID NO:1. This method of screening a molecule for the ability to bind a known primary ligand can also be

performed in vitro in, for example, a test tube or well, as discussed above for the in vitro screening of a peptide for the ability to bind a known primary ligand.

The invention further provides a green fluorescent protein complex which comprises a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide which reversibly binds a primary ligand, the primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at the second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. When the green fluorescent protein complex is excited at the first wavelength, the amount of fluorescence emission at the third wavelength is reduced when the primary ligand-binding peptide is bound to primary ligand as compared to the amount of fluorescence emission at the third wavelength when the primary ligand-binding peptide is not bound to the primary ligand. Also provided is a cell which expresses the green fluorescent protein complex. Suitable cells include bacterial and mammalian cells, including *Escherichia coli* cells.

Preferably, the primary ligand is calmodulin and the primary ligand-binding peptide is a calmodulin-binding peptide, such as the peptide having the amino acid sequence as shown in SEQ ID NO:1. The first green fluorescent protein is preferably a blue-shifted green fluorescent protein (which is excited at a first wavelength maxima of 380 nm and emits fluorescence at a second wavelength maxima of 440 nm and emits the second green fluorescent protein is preferably a red-shifted green fluorescent protein (which is excited at the second wavelength maxima of 495 nm and emits fluorescence at a third wavelength maxima of 505 nm). In one embodiment, the green fluorescent protein complex may further comprise the primary ligand covalently attached to one of the first green fluorescent protein or the second green fluorescent protein so as to provide for detection of a second ligand. Here the sensitivity of the indicator to the secondary ligand can be modified by altering the interface between the primary ligand and the primary ligand-binding peptide.

Standard techniques can be used to replace the GFPs of plasmid pFIP-CB<sub>SM</sub> with alternative GFPs, and standard techniques can be used to replace the calmodulin-binding



peptide of plasmid pFIP-CB<sub>SM</sub> with alternative primary ligand-binding peptides (or a peptide to be screened for primary ligand-binding activity). Generally, this involves the use of restriction enzymes and ligation (see below).

The green fluorescent protein complex according to the subject invention can be introduced into a cell. Techniques for introducing the complex or a deoxyribonucleic acid molecule in the form of a plasmid allowing expression of the complex into a cell are known in the art. These include: 1) microinjection, in which the purified complex or RNA is injected directly into the cell through fine glass needles; 2) dextran incubation, in which DNA is incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell; 3) calcium phosphate coprecipitation, in which cells efficiently take in DNA in the form of a precipitate with calcium phosphate; 4) electroporation, in which cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage through these vesicles may sometimes destroy or damage DNA); 5) liposomal mediated transformation, in which DNA is incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm; 6) biolistic transformation, in which DNA is absorbed to the surface of gold particles and fired into cells under high pressure using a ballistic device; and 7) viral-mediated transformation, in which nucleic acid molecules are introduced into cells using viral vectors. Since viral growth depends on the ability to get the viral genome into cells, viruses have devised efficient methods for doing so. These viruses include retroviruses and lentivirus, adenovirus, herpesvirus, and adeno-associated virus. Preferably, the plasmid is introduced into a cell by microinjection or the use of a vector (a plasmid or viral vector, for example). U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase.

These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

5           The methods of the subject invention use fluorescence resonance energy transfer (FRET) between spectral variants of GFP to monitor primary ligands or secondary ligands. FRET is described in more detail in Mitra et al. 1996. Briefly, FRET is a process in which an excited fluorophore (the donor) transfers its excited state energy to a light absorbing molecule (the acceptor). Generally, the fluorescence of the donor  
10       decreases as the fluorescence of the acceptor increases and vice versa.

### EXAMPLE I

The  $\text{Ca}^{2+}$ -binding protein, calmodulin, is a key transducer of intracellular  $\text{Ca}^{2+}$  ion signals, mainly through  $\text{Ca}^{2+}$ -dependent modulation of numerous enzyme activities  
15       (Bredt and Snyder 1990; Cho et al. 1992; Edelman et al. 1996; Crivici and Ikura 1995; Kink et al. 1990; Lu and Means 1993; Manalan and Klee 1984; Means et al. 1991; Wang et al. 1996). This example describes a fluorescent indicator protein whose emission changes reversibly from green to blue light when it binds  $(\text{Ca}^{2+})_4$ -calmodulin ( $K_d=0.4$  nM), in analogy with a calmodulin-dependent enzyme activity. This response  
20       has been monitored in cells microinjected with the indicator. It closely mirrors changes in the intracellular free  $\text{Ca}^{2+}$  ion concentration, responding to a rapid, receptor-mediated, increase with no discernable lag ( $<300$  ms). The fractional indicator response that can be achieved in the absence of co-injected calmodulin is consistent with a free intracellular  $(\text{Ca}^{2+})_4$ -calmodulin concentration of  $\sim 1$  nM. Images using green/blue  
25       fluorescence ratios demonstrate the utility of this indicator for investigating spatio-temporal changes in  $(\text{Ca}^{2+})_4$ -calmodulin levels in living cells.

The fluorescent indicator protein (FIP- $\text{CB}_{\text{SM}}$ ) contains two previously characterized green fluorescent protein (GFP) variants; one with fluorescence excitation and emission maxima of  $\sim 380$  and  $\sim 440$  nm (BGFP; Heim et al. 1994), the other with  
30       excitation and emission maxima of  $\sim 495$  and  $\sim 505$  nm (RGFP; Delegrave et al. 1995). These are joined by a linker sequence containing the calmodulin-binding domain of avian smooth muscle myosin light chain kinase (Fig. 1). When excited at 380 nm, FIP- $\text{CB}_{\text{SM}}$  exhibits a pronounced emission peak at 505 nm, which is reduced by 65% when

Ca<sup>2+</sup>-calmodulin is bound to the linker. There is a corresponding increase in F<sub>440</sub> so that the F<sub>440</sub>/F<sub>505</sub> emission ratio increases from a value of 0.6 to a value of 3 when FIP-CB<sub>SM</sub> is fully saturated with calmodulin. This behavior is consistent with a calmodulin-dependent reduction in FRET between the fluorophores in the two GFP domains in FIP-CB<sub>SM</sub> (Fig. 2). This is the first example of a fusion between two GFPs that exhibits reversible changes in inter-fluorophore FRET in response to a ligand. The fluorescence properties of FIP-CB<sub>SM</sub> make it suitable for single-wavelength and emission-ratio measurements. FIP-CB<sub>SM</sub> binds (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin with a K<sub>d</sub> of 0.4 nM, which is close to the 1 nM apparent value inferred for the complex between calmodulin and smooth muscle myosin light chain kinase (Figs. 3 and 4) (Adelstein et al. 1981).

Purified GFP is known to dimerize in solution, and crystallographic data suggest that the two subunits are in an antiparallel orientation, placing the N-terminus of one protein about 70 Å from the C-terminus of the other (Ormo et al. 1996; Yang et al. 1996). Modeling studies suggest that the linker in FIP-CB<sub>SM</sub> should allow the two GFP domains to associate similarly. The modest ionic strength dependence of the FIP-CB<sub>SM</sub> emission spectrum measured in the absence of calmodulin may reflect the presence of stabilizing electrostatic interactions between amino acids at the interface between the two GFP domains. Perhaps because of changes in the orientation between the two GFP domains, the efficiency of FRET between the fluorophores in FIP-CB<sub>SM</sub> appears to depend upon the length of the linker sequence. The F<sub>440</sub>/F<sub>505</sub> ratio increases from a value of 0.6 with the 26 amino acid linker in FIP-CB<sub>SM</sub>, to 0.9 and 1.5 with 20 and 11 amino acid linkers, respectively. As seen in the crystal structure, the two chromophores in a GFP dimer are ~25 Å apart (Yang et al. 1996). The complex between (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin and the smooth muscle myosin light chain kinase calmodulin-binding domain forms a globular structure 40 Å in diameter (Crivici and Ikura 1995). When calmodulin binds to the kinase calmodulin-binding domain in the FIP-CB<sub>SM</sub> linker, it adopts an α-helical conformation. In this conformation the 17 residue sequence has a length of ~30 Å, about half its length in an extended conformation. Thus, when (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin is bound to the linker in FIP-CB<sub>SM</sub>, the distance between the fluorophores is likely to increase from ~25 Å to ~65 Å, with calmodulin tightly sandwiched between the two GFP domains (Fig. 1). The efficiency of FRET depends upon the distance between the donor and acceptor fluorophores and upon the relative orientation of their dipole moments (Stryer 1978). Changes in the distance and possibly also the orientation

parameters is clearly the basis for the observed calmodulin-dependent change in the FIP-CB<sub>SM</sub> fluorescence emission spectrum. In attempting to design FIPs with novel specificities, it is important to bear in mind that shortening of the linker sequence in FIP-CB<sub>SM</sub> when (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin is bound may paradoxically help to force the GFP domains apart.

Purified FIP-CB<sub>SM</sub> has been microinjected into HEK-293 cells stably transfected with epitope-tagged thyrotropin releasing hormone (TRH) receptor, a Ca<sup>2+</sup>-mobilizing G-protein-coupled receptor (Nelson and Hinkle 1994). The response of the FIP-CB<sub>SM</sub> in these cells to externally applied TRH, ionomycin and 1,2-

*bis*(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was measured. The concentration of the FIP-CB<sub>SM</sub> solution used in microinjection experiments was 80 μM, and an estimate of intracellular FIP-CB<sub>SM</sub> is in a concentration range of 1-10 μM in microinjected cells, similar to estimates for the intracellular concentrations of high-abundance calmodulin targets, including smooth muscle myosin light chain kinase, calcineurin, calmodulin-dependent protein kinase II and cerebellar nitric oxide synthase (Tansey et al. 1994). When FIP-CB<sub>SM</sub> fluorescence is excited at 380 nm, images of whole cells using an F<sub>510</sub>/F<sub>440</sub> emission ratio and time courses for relative changes in whole-cell F<sub>510</sub> exhibit striking responses to changes in the intracellular Ca<sup>2+</sup> ion concentration (Figs. 6-11). The results suggest no discernable lag (<300 ms) between activation of TRH receptor and formation of significant levels of (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin-target complexes in the nucleus or cytoplasm (Figs. 6-11).

To estimate the maximal intracellular FIP-CB<sub>SM</sub> response, cells were injected with a 1:1 mole ratio of FIP-CB<sub>SM</sub> and calmodulin. In spite of the higher and more sustained Ca<sup>2+</sup> levels caused by ionomycin treatment, both ionomycin and TRH elicit a similar FIP-CB<sub>SM</sub> response in these cells, suggesting that is near the maximum (Figs. 6 and 7). The average decrease in F<sub>510</sub> caused by ionomycin or TRH is ~30%. The average ionomycin-dependent decrease in F<sub>510</sub> seen in cells injected with FIP-CB<sub>SM</sub> alone is ~10% (Figs. 7 and 8). This difference between cells injected with FIP-CB<sub>SM</sub> alone and those injected with FIP-CB<sub>SM</sub>/calmodulin appears to be independent of the intracellular FIP-CB<sub>SM</sub> concentration as estimated from the area-normalized F<sub>510</sub> of resting cells, which varies by a factor of 8 among different cells. The lower FIP-CB<sub>SM</sub> response exhibited in cells injected with the indicator alone suggests a limiting free (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin concentration in the cell. This is also consistent with the

significantly greater reduction in  $F_{510}$  caused by ionomycin treatment of these cells, compared with that caused by TRH treatment, since the higher and more sustained  $\text{Ca}^{2+}$  ion levels resulting from ionomycin treatment would be expected to lead to higher levels of  $(\text{Ca}^{2+})_4$ -calmodulin (Figs. 6-11).

5        Assuming that the  $F_{510}$  value measured in the presence of BAPTA represents completely unliganded FIP- $\text{CB}_{\text{SM}}$ , and that  $F_{510}$  value measured in ionomycin treated cells injected with FIP- $\text{CB}_{\text{SM}}$ /calmodulin represents fully liganded FIP- $\text{CB}_{\text{SM}}$ , then the fractional response to ionomycin seen with cells injected only with FIP- $\text{CB}_{\text{SM}}$  corresponds to a free  $(\text{Ca}^{2+})_4$ -calmodulin level of 0.2 nM. Treatment with BAPTA can  
10        cause  $F_{510}$  in cells injected with FIP- $\text{CB}_{\text{SM}}$  or FIP- $\text{CB}_{\text{SM}}$ /calmodulin to increase above the value measured at the start of the experiment (Figs. 10 and 11). This indicates a significant basal level of  $(\text{Ca}^{2+})_4$ -calmodulin, which is unfortunately difficult to quantitate. However, the 30% reduction in  $F_{510}$  measured in cells microinjected with FIP- $\text{CB}_{\text{SM}}$ /calmodulin represents ~50% of the indicator's dynamic range, so the basal  
15         $(\text{Ca}^{2+})_4$ -calmodulin concentration in cells is unlikely to exceed 0.4 nM (Fig. 2). One can therefore conservatively estimate that the average free  $(\text{Ca}^{2+})_4$ -calmodulin level increases to ~1 nM in microinjected cells treated with ionomycin. As with any intracellular indicator, FIP- $\text{CB}_{\text{SM}}$  undoubtedly affects homeostasis of the ligand it is designed to detect:  $(\text{Ca}^{2+})_4$ -calmodulin.

20        A low maximal level of  $(\text{Ca}^{2+})_4$ -calmodulin in cells is consistent with mobility studies of microinjected tagged calmodulin, which indicate that >95% of the total calmodulin is bound to other intracellular proteins at a saturating free  $\text{Ca}^{2+}$  ion concentration. A low maximal level of  $(\text{Ca}^{2+})_4$ -calmodulin also suggests that for a typical target, with a 1 nM dissociation constant for  $(\text{Ca}^{2+})_4$ -calmodulin, changes in  
25        calmodulin-binding affinity will result in proportional changes in calmodulin-dependent target activity. In agreement with this, Stull et al. (1993) have demonstrated that a 10-fold decrease in the affinity of smooth muscle myosin light chain kinase for  $(\text{Ca}^{2+})_4$ -calmodulin significantly reduces levels of this enzyme activity in smooth muscle cells.

30        Cells injected cytoplasmically with FIP- $\text{CB}_{\text{SM}}$  exhibit a diffuse fluorescence that is excluded from the nucleus and other cellular organelles. Imaging using the  $F_{510}/F_{440}$  ratio shows an obvious  $\text{Ca}^{2+}$ -dependent FIP- $\text{CB}_{\text{SM}}$  response in cells injected either with the FIP- $\text{CB}_{\text{SM}}$  alone or with FIP- $\text{CB}_{\text{SM}}$ /calmodulin. A clear FIP- $\text{CB}_{\text{SM}}$  response is observed in both cytoplasm and nucleus. It has been suggested that calmodulin plays an

important role in controlling events in the nucleus, including mitosis (Lu et al. 1993; Wang et al. 1996). The results certainly indicate that comparable levels of free  $(\text{Ca}^{2+})_4$ -calmodulin are achieved in the nucleus and cytosol. There appear to be regional variations in the FIP-CB<sub>SM</sub> response that may represent a heterogeneous distribution of calmodulin and/or free  $\text{Ca}^{2+}$  ion in the cell.

## EXAMPLE II

Fig. 2 shows the calmodulin-dependent changes in the FIP-CB<sub>SM</sub> fluorescence emission spectrum. FIP-CB<sub>SM</sub> at a concentration of 1  $\mu\text{M}$  was titrated with 0.2  $\mu\text{M}$  increments of calmodulin in the presence of 300  $\mu\text{M}$   $\text{CaCl}_2$ . The buffer also contained 25 mM Tris-HCl, pH 7.5 and 0.1 M NaCl. Fluorescence was excited at 380 nm. The effects of calmodulin on the FIP-CB<sub>SM</sub> emission spectra are completely reversed by 5 mM EDTA. A scheme depicting the conformational change undergone by FIP-CB<sub>SM</sub> upon binding  $(\text{Ca}^{2+})_4$ -calmodulin is presented in Fig. 1. The RGFP (R) and BGFP (B) domains in FIP-CB<sub>SM</sub> are joined by the linker sequence: SEQ ID NO:1: GTSSRRKWNKTGHAVRAIGRLSSTGA, which contains the calmodulin-binding domain from avian smooth muscle myosin light chain kinase (Guerriero et al. 1986), shown in boldface type. The fluorophores in the GFP domains are represented by shaded rectangles. The shape used for the GFP domains is based upon the published crystal structures for GFP, which indicate an eleven-stranded  $\beta$ -barrel (Ormo et al. 1996; Yang et al. 1996). Based upon the corresponding calmodulin-peptide structure, the linker sequence in FIP-CB<sub>SM</sub> is enfolded by the two lobes of  $(\text{Ca}^{2+})_4$ -calmodulin and adopts an  $\alpha$ -helical conformation. Calmodulin is depicted as two hemispheres, corresponding to the lobes, joined by the flexible central helix tether (Persechini and Kretsinger 1988). The two  $\text{Ca}^{2+}$  ions bound to each lobe in  $(\text{Ca}^{2+})_4$ -calmodulin are depicted as filled circles. FIP-CB<sub>SM</sub> was expressed in *E. coli* and purified essentially as described by Mitra et al. (1996). The purified protein exhibits optical absorbance maxima at 480 and 380 nm with respective  $\epsilon$  values of 89 and 36  $\text{mM}^{-1}\text{cm}^{-1}$ , which correspond with the absorbances of RGFP and BGFP, respectively. The 380 nm-excited fluorescence emission spectrum of a control protein in which the linker sequence used in FIP-CB<sub>SM</sub> is replaced by the sequence: SEQ ID NO:2: GTSSGSSTTGA is unaffected by  $(\text{Ca}^{2+})_4$ -calmodulin levels as high as 3  $\mu\text{M}$ . The FIP-CB<sub>SM</sub> emission spectrum is independent of pH between 7.0 and 8.0, either in the presence or absence of bound

calmodulin. The emission spectrum of FIP-CB<sub>SM</sub> is affected by changes in ionic strength; the  $F_{440}/F_{505}$  emission ratio increases by 0.1 with each 50 mM increment in ionic strength between 130 and 300 mM. In the presence of a saturating level of  $(Ca^{2+})_4$ -calmodulin the  $F_{440}/F_{505}$  ratio of FIP-CB<sub>SM</sub> is unaffected by ionic strength changes in this range.

### EXAMPLE III

Figs. 3-5 show the characterization of calmodulin binding by FIP-CB<sub>SM</sub>. Fig. 3 shows the titration of a mixture of 8.2 nM FIP-CB<sub>SM</sub> and 200 nM calmodulin with increasing concentrations of free  $Ca^{2+}$  ion. Free  $Ca^{2+}$  ion concentrations were established by incremental additions of standard  $CaCl_2$  solutions to a buffer containing 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.5 mM  $MgCl_2$ , and 3 mM 1,2-bis(o-amino-5,5'-dibromophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid ( $Br_2$ BAPTA) at 25°C. Values for the free  $Ca^{2+}$  concentration given in the figure were calculated using the MaxChelator software package (Bers et al. 1994). The curve shown in the figure was calculated using an equation of the form:  $F = \alpha (L^n)/(L^n + K_a)$  where  $F$  is the fractional saturation of FIP-CB<sub>SM</sub> with calmodulin, given in the figure as  $1 - [(F - F_{min})/(F_{max} - F_{min})]$ ,  $L$  is the free  $Ca^{2+}$  ligand concentration,  $K_a$  is an apparent dissociation constant that depends upon the total amount of calmodulin, and  $n$  is the number of interacting sites, which equaled 3.9 for the curve shown. This indicates that  $(Ca^{2+})_4$ -calmodulin is the species bound by FIP-CB<sub>SM</sub>.  $F$  is the fluorescence measured at 505 nm,  $F_{max}$  and  $F_{min}$  are the values for  $F$  measured at maximal and minimal free  $Ca^{2+}$  ion concentrations.  $\alpha$  is a correction factor allowing adjustment of the maximal fraction of FIP-CB<sub>SM</sub> bound to give the best fit.  $\alpha$  values are  $<1.1$  for the curves shown in panels A and B.

Fig. 4 shows the binding of  $(Ca^{2+})_4$ -calmodulin to FIP-CB<sub>SM</sub> at concentrations of 1 (■) and 1.4 (▲) nM. Buffer conditions are described above for Fig. 2 (Example II). The curve fitting data measured at a 1 nM FIP-CB<sub>SM</sub> concentration was generated according to a standard single-site kinetic model. The curve fitting data measured at a 1.4 nM FIP-CB<sub>SM</sub> concentration was generated according to an equation of the form:  $F = \alpha \{ (P_t + L_t + K_d) - [(P_t + L_t + K_d)^2 - 4(P_t)(L_t)]^{0.5} \} / 2(P_t)$ , where  $L_t$  is the total calmodulin concentration and  $P_t$  is the total FIP-CB<sub>SM</sub> concentration. In both cases the curves fitting the data were generated using a  $K_d$  value of 0.4 nM. Emission spectra for titration of FIP-CB<sub>SM</sub> at a 1.4 nM concentration are shown in Fig. 5.

#### EXAMPLE IV

Figs. 6-11 show time courses for area-normalized enhanced fluorescence in cells microinjected with FIP-CB<sub>SM</sub>. FIP-CB<sub>SM</sub> fluorescence was excited at 380 nm and measured at 510 nm. Data are presented as simple fraction of the F<sub>510</sub> measured at the start of the experiment (F<sub>0</sub>), except for fura-2 measurements, which are presented as 340/380 fluorescence excitation ratios, determined as previously described. All traces are averages of data collected from 8-15 cells. B, T, C and I indicate external applications of 3 mM BAPTA, 1  $\mu$ M TRH, 3 mM CaCl<sub>2</sub> and 3  $\mu$ M ionomycin. Figs. 6 and 7 contain traces for cells injected with FIP-CB<sub>SM</sub> and calmodulin in a 1:1 mole ratio. Traces for cells injected with FIP-CB<sub>SM</sub> alone are presented in Figs. 8 and 9. Figs. 10 and 11 contain traces for the fura-2 response of mock-injected cells. It was found that microinjection of a synthetic peptide based on the calmodulin-binding domain in skeletal muscle myosin light chain kinase (Blumenthal and Krebs 1987) at a concentration identical to that used for FIP-CB<sub>SM</sub> has no discernable effect on the fura-2 response. This was also found to be the case for cells microinjected with concentrations of calmodulin comparable to those co-injected with FIP-CB<sub>SM</sub>. Cells microinjected with a control GFP fusion protein (see above description of Fig. 2; Example II), either alone or in a 1:1 mole ratio with calmodulin, exhibited no significant Ca<sup>2+</sup>-dependent changes in F<sub>510</sub>. The gap in the trace presented in Fig. 7 is due to a camera malfunction.

#### EXAMPLE V

A second fluorescent indicator protein (FIP-CA<sub>3</sub>) useful for monitoring levels of secondary ligands is shown in Fig. 12. In this embodiment, the calmodulin protein (the primary ligand) is covalently attached to the green fluorescent protein complex described in the above examples. FRET in this embodiment is dependent only on Ca<sup>2+</sup> (a secondary ligand) levels.

Fig. 13 shows the time course for area-normalized enhanced fluorescence in cells microinjected with FIP-CA<sub>3</sub> (comparable to Figs. 6-11 for FIP-CB<sub>SM</sub>).

Fig. 14 characterizes the calcium binding by FIP-CA<sub>3</sub>, showing the titration of FIP-CA<sub>3</sub> with increasing concentrations of free Ca<sup>2+</sup> ion.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the



invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table 1 Primary Sequences of some known and putative calmodulin binding domains of protein and peptide calmodulin targets

Target*	Sequence <sup>b</sup> (with SEQ ID NO: in parentheses)	Reference
smMLCK (M13) (c)	K R R R W K K N F I A V S A A N R F F K K I S S S G A L (3)	Blumenthal et al. 1987
smMLCK (smMLCKp)	A R R K W Q K T G H A V R A I G R L S S (4)	Lowenstein et al. 1992
CaMKII	A R R K L K K G A I L T T M L A T R N F S (5)	Novack et al. 1991
Caldesmon	G V R N I K S M W E K G N V F S S (6)	Zhang et al. 1994
Calspermin	A R R K L K A A V K A V A S S R L G S (7)	Payne et al. 1988
PFK (M11)	F M N N W E V Y K L L A I R P P A P K S G S Y T V (8)	Buschmeier et al. 1987
Calcineurin	A R K E V I R N K I R A I G K M A R V F S V L R (9)	Kincaid et al. 1988
PhK (PhK5)	L R R L I D A Y A F R I Y G H W V K K Q Q N R G (10)	Dasgupta et al. 1989
(PhK13)	R G K F K V I C L T V L A S V R I Y Y Q Y R R V K P G (11)	Dasgupta et al. 1989
Ca <sup>2+</sup> -ATPase (C28W)	L R R G Q I L W F R G L N R I Q T Q I K V V N A F S S (12)	Vorherr et al. 1990
59-kDa PDE	R R K H L Q R P I F R L R C L V K Q L E K (13)	Vorherr et al. 1993
60-kDa PDE	T E K M H Q R L K G I L R C L V K Q L E K (14)	Novack et al. 1991
NOS (NO-30)	K R R A I G E K K L A E A V K F S A K L M G Q (15)	Vorherr et al. 1993
Type I AC (AC-28)	I-K P A K R M K I D L L W K I A R A G A R S A V G T E A (16)	Vorherr et al. 1993
Bordetella pertussis AC	K A H K A A T K I Q A S F R L M H T V A T F N S I K E (17)	Oldenburg et al. 1992
Neuromodulin	K T A S P M K S A R L K S F K L S G F S F K K S K K (18)	Chapman et al. 1991
Spectrin	K K K K K F S F K K P F K L S G F S F K K S K K (19)	Leto et al. 1989
MARCKS	K K K K K F S F K K P F K L S G F S F K K S K K (20)	Graff et al. 1991
F52 or MacMARCKS	K Q Q X E K T R L N T P N T Y L R V N V A D E V Q R N M G S (22)	Blackshear et al. 1992
$\beta$ -Adducin	K D Q V A N R L R D L L I V K R I V E L L G R R (24)	Scaramuzzino 1993
HSP90 $\alpha$	I H R L R D L L I V K R I V E L L G R R (25)	Minami et al. 1993
HIV-1 gp160	Q Q L A T L I Q K T Y R G W R C R T H Y Q L M (26)	Srinivas et al. 1993
BBMHCI	R A A C I R I N L K A L A A L A K K I L (27)	Mercer et al. 1991
Dilute MHC	G I G A V L K V L T T G L P A L I S W I K R K R Q Q (28)	Mercer et al. 1991
Mastoparan	H S Q G T F T T S D Y S K Y L D S R R A Q D F V Q W L M N T (29)	Malencik et al. 1983b
Melittin	H S D G T F T S E L S R L R D S A R L Q R L Q G L V (30)	Malencik et al. 1983a
Glucagon	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N (31)	Malencik et al. 1983a
Secretin	Y A D G T F I S D Y S A I M N K I R Q Q D F V N W L L A Q Q K S (32)	Malencik et al. 1983a
VIP	K L W K K L L K L L K L L K L L G (33)	DeGrado et al. 1985
GIP		
Model peptide CBP2		

\* Abbreviations: AC, adenylyl cyclase; BBMHCI, brush-border myosin heavy chain-I; CaMKII, calmodulin kinase II; CBP2, calmodulin binding peptide-2; GIP, gastrin inhibitory peptide; HIV-1 gp160, human immunodeficiency virus envelope glycoprotein 160; HSP, heat-shock protein; MARCKS, myristoylated alanine-rich C kinase substrate; MHC, myosin heavy chain; NOS, nitric oxide synthase; PDE, phosphodiesterase; PFK, phosphofructokinase; PhK, phosphorylase kinase; sk-, smMLCK, skeletal muscle- and smooth muscle-myosin light chain kinase; VIP, vasoactive intestinal peptide.

<sup>b</sup> Alignment of the CaM domains was made by visual inspection based on alignment of the putatively conserved major (bold and underlined) and minor (bold) hydrophobic anchors that interact with the hydrophobic patches of the C- and N-terminal domains of CaM (Ikura et al. 1992), and on the alignment of the conserved basic residue (bold and italicized) analogous to that residue of MLCK that is required for activation by CaM (Meador et al. 1992; Meador et al. 1993). Precise boundaries of the CaM-binding domain are not known for all targets.

<sup>c</sup> Names in parentheses are those used in the literature for the synthetic peptides containing the sequences listed.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: University of Rochester
- (ii) TITLE OF INVENTION: DETECTION BY FRET CHANGES OF  
LIGAND BINDING BY GFP FUSION  
PROTEINS
- (iii) NUMBER OF SEQUENCES: 33
- (iv) CORRESPONDENCE ADDRESS:
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  - (D) STATE: New York
  - (E) COUNTRY: USA
  - (F) ZIP: 14603
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version
- #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/842,322
  - (B) FILING DATE: 24-APR-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Goldman, Michael L.
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  - (C) REFERENCE/DOCKET NUMBER: 176/60171
- (ix) TELECOMMUNICATION INFORMATION:
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Thr Ser Ser Arg Arg Lys Trp Asn Lys Thr Gly His Ala Val Arg  
1 5 10 15  
Ala Ile Gly Arg Leu Ser Ser Thr Gly Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Thr Ser Ser Gly Ser Ser Thr Thr Gly Ala  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Arg Arg Trp Lys Lys Asn Phe Ile Ala Val Ser Ala Ala Asn Arg  
1 5 10 15  
Phe Lys Lys Ile Ser Ser Ser Gly Ala Leu  
20 25

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Arg Arg Lys Trp Gln Lys Thr Gly His Ala Val Arg Ala Ile Gly  
1                      5                      10                      15  
  
Arg Leu Ser Ser  
                    20

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Arg Arg Lys Leu Lys Gly Ala Ile Leu Thr Thr Met Leu Ala Thr  
1                      5                      10                      15  
  
Arg Asn Phe Ser  
                    20

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Val Arg Asn Ile Lys Ser Met Trp Glu Lys Gly Asn Val Phe Ser  
1                      5                      10                      15  
  
Ser

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant



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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Arg Arg Lys Leu Lys Ala Ala Val Lys Ala Val Val Ala Ser Ser  
1 5 10 15

Arg Leu Gly Ser  
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Phe Met Asn Asn Trp Glu Val Tyr Lys Leu Leu Ala His Ile Arg Pro  
1 5 10 15

Pro Ala Pro Lys Ser Gly Ser Tyr Thr Val  
20 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Arg Lys Glu Val Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met  
1 5 10 15

Ala Arg Val Phe Ser Val Leu Arg  
20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Leu Arg Arg Leu Ile Asp Ala Tyr Ala Phe Arg Ile Tyr Gly His Trp
1           5           10           15

Val Lys Lys Gly Gln Gln Gln Asn Arg Gly
          20           25

```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Arg Gly Lys Phe Lys Val Ile Cys Leu Thr Val Leu Ala Ser Val Arg
1           5           10           15

Ile Tyr Tyr Gln Tyr Arg Arg Val Lys Pro Gly
          20           25

```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Leu Arg Arg Gly Gln Ile Leu Trp Phe Arg Gly Leu Asn Arg Ile Gln
1           5           10           15

Thr Gln Ile Lys Val Val Asn Ala Phe Ser Ser Ser
          20           25

```

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## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Arg Lys His Leu Gln Arg Pro Ile Phe Arg Leu Arg Cys Leu Val  
1                      5                      10                      15  
  
Lys Gln Leu Glu Lys  
                    20

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Thr Glu Lys Met Trp Gln Arg Leu Lys Gly Ile Leu Arg Cys Leu Val  
1                      5                      10                      15  
  
Lys Gln Leu Glu Lys  
                    20

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Arg Arg Ala Ile Gly Phe Lys Lys Leu Ala Glu Ala Val Lys Phe  
1                      5                      10                      15  
  
Ser Ala Lys Leu Met Gly Gln

20

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile	Lys	Pro	Ala	Lys	Arg	Met	Lys	Phe	Lys	Thr	Val	Cys	Tyr	Leu	Leu
1				5					10					15	
Val	Gln	Leu	Met	His	Cys	Arg	Lys	Met	Phe	Lys	Ala				
			20					25							

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ile	Asp	Leu	Leu	Trp	Lys	Ile	Ala	Arg	Ala	Gly	Ala	Arg	Ser	Ala	Val
1				5					10					15	
Gly	Thr	Glu	Ala												
			20												

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Ala His Lys Ala Ala Thr Lys Ile Gln Ala Ser Phe Arg Gly His

Ile Thr Arg Lys Lys Leu Lys Gly Glu Lys Lys

          20                    25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Lys Thr Ala Ser Pro Trp Lys Ser Ala Arg Leu Met Val His Thr Val  
1 5 10 15

Ala Thr Phe Asn Ser Ile Lys Glu  
20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Lys Lys Lys Lys Lys Arg Phe Ser Phe Lys Lys Ser Phe Lys Leu Ser  
1 5 10 15

Gly Phe Ser Phe Lys Lys Ser Lys Lys  
20 25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

BNSDOCID: <WO\_9848278A1 I >

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Lys Lys Lys Lys Lys Phe Ser Phe Lys Lys Pro Phe Lys Leu Ser Gly  
1 5 10 15  
Leu Ser Phe Lys Arg Asn Arg Lys  
20

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Lys Gln Gln Lys Glu Lys Thr Arg Trp Leu Asn Thr Pro Asn Thr Tyr  
1 5 10 15  
Leu Arg Val Asn Val Ala Asp Glu Val Gln Arg Asn Met Gly Ser  
20 25 30

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Asp Gln Val Ala Asn Ser Ala Phe Gln Glu Arg Leu Arg Lys His  
1 5 10 15  
Gly Leu Glu Val Ile  
20

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Tyr	His	Arg	Leu	Arg	Asp	Leu	Leu	Leu	Ile	Val	Lys	Arg	Ile	Val	Glu
1				5					10					15	
Leu	Leu	Gly	Arg	Arg											
				20											

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gln	Gln	Leu	Ala	Thr	Leu	Ile	Gln	Lys	Thr	Tyr	Arg	Gly	Trp	Arg	Cys
1				5					10					15	
Arg	Thr	His	Tyr	Gln	Leu	Met									
				20											

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Arg	Ala	Ala	Cys	Ile	Arg	Ile	Gln	Lys	Thr	Ile	Arg	Gly	Trp	Leu	Leu
1				5					10					15	
Arg	Lys	Arg	Tyr	Leu	Cys	Met	Gln								
				20											

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid

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- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu  
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu  
1 5 10 15  
Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln  
20 25

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

His Ser Gln Gly Thr Phe Thr Thr Ser Asp Tyr Ser Lys Tyr Leu Asp  
1 5 10 15  
Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr  
20 25 30

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 amino acids
  - (B) TYPE: amino acid



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(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg	Asp	Ser
1				5				10						15	

Ala	Arg	Leu	Gln	Arg	Leu	Leu	Gln	Gly	Leu	Val
		20					25			

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln
1				5				10						15	

Met	Ala	Val	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn
			20				25				

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Tyr	Ala	Asp	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ala	Ile	Met	Asn	Lys
1				5				10						15	

Ile	Arg	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala	Gln	Gln	Gln	Lys
		20					25					30			

Ser

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## (2) INFORMATION FOR SEQ ID NO:33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Lys	Leu	Trp	Lys	Lys	Leu	Leu	Lys	Leu	Leu	Lys	Lys	Leu	Leu	Lys	Leu
1				5				10					15		

Gly

**WHAT IS CLAIMED IS:**

1. A method of monitoring the amount of a primary ligand in a cell, said method comprising:

constructing a green fluorescent protein complex, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein;

introducing said green fluorescent protein complex into a cell;

determining a base amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength;

determining amount of fluorescence emission over time at said third wavelength when said cell is excited over time at said first wavelength; and

comparing said amount of fluorescence emission over time to said base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates presence of said primary ligand in said cell and wherein changes in said amount of fluorescence emission over time indicate changes in the amount of said primary ligand in said cell over time.

2. The method of claim 1 further comprising quantitating the amount of fluorescence emission over time to estimate the amount of said primary ligand in said cell over time.

3. The method of claim 1 wherein said primary ligand is calmodulin and said primary ligand-binding peptide is a calmodulin-binding peptide.

4. The method of claim 3 wherein said calmodulin-binding peptide has an amino acid sequence as shown in SEQ ID NO:1.

5. The method of claim 1 wherein the first green fluorescent protein is a blue-shifted green fluorescent protein.

6. The method of claim 5 wherein the blue-shifted green fluorescent protein is excited at a first wavelength maxima of 380 nm and emits fluorescence at a second wavelength maxima of 440 nm.

7. The method of claim 1 wherein the second green fluorescent protein is a red-shifted green fluorescent protein.

8. The method of claim 7 wherein said red-shifted green fluorescent protein is excited at said second wavelength maxima of 495 nm and emits fluorescence at a third wavelength maxima of 505 nm.

9. The method of claim 1 further comprising introducing into the cell a secondary ligand which binds to said primary ligand and which is necessary for binding of said primary ligand to said primary ligand-binding peptide.

10. The method of claim 9 wherein said primary ligand is calmodulin and said secondary ligand is  $\text{Ca}^{2+}$ .

11. The method of claim 1 for further monitoring the amount of a secondary ligand in the cell, where said secondary ligand binds to said primary ligand and is necessary for binding of said primary ligand to said primary ligand-binding peptide, said method further comprising:

introducing said primary ligand into the cell prior to determining the base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates presence of the secondary ligand in said cell and wherein changes in said amount of fluorescence emission over time indicates changes in the amount of said secondary ligand in said cell over time.

12. The method of claim 11 wherein introducing said primary ligand comprises covalently attaching said primary ligand to one of said first green fluorescent

protein or said second green fluorescent protein in said green fluorescent protein complex.

13. The method of claim 11 wherein said secondary ligand is  $\text{Ca}^{2+}$  and said primary ligand is calmodulin.

14. The method of claim 11 wherein said secondary ligand is a metal ion.

15. The method of claim 14 wherein said metal ion is selected from the group consisting of manganese, nickel, and cadmium.

16. The method of claim 11 wherein said secondary ligand is a molecule.

17. A method of screening a peptide for primary ligand-binding activity, said method comprising:

constructing a green fluorescent protein complex, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein;

introducing said green fluorescent protein complex into a cell in the absence of said primary ligand;

determining a base amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength;

adding said primary ligand to said cell;

determining amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates that said peptide has primary ligand-binding activity.

18. The method of claim 17 wherein said primary ligand is calmodulin.

19. A method of screening a molecule for the ability to bind a primary ligand, said method comprising:

constructing a green fluorescent protein complex, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein;

introducing said green fluorescent protein complex into a cell with said primary ligand;

determining a base amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength;

adding a molecule to said cell;

determining amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission greater than said base amount of fluorescence emission indicates that said molecule has primary ligand-binding activity.

20. The method of claim 19 wherein said primary ligand is calmodulin.

21. A method of screening a peptide for primary ligand-binding activity, said method comprising:

expressing a green fluorescent protein complex in a cell, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein;

purifying the expressed green fluorescent protein complex and placing said purified green fluorescent protein complex in a test vesicle;

determining a base amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength;

adding said primary ligand to said test vesicle;

determining amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates that said peptide has primary ligand-binding activity.

22. The method of claim 21 wherein said primary ligand is calmodulin.

23. The method of claim 21 wherein said purifying comprises affinity chromatography.

24. The method of claim 21 wherein said test vesicle is a test tube or test well.

25. A method of screening a molecule for primary ligand-binding activity, said method comprising:

expressing a green fluorescent protein complex in a cell, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein;

purifying the expressed green fluorescent protein complex and placing said purified green fluorescent protein complex in a test vesicle;

adding said primary ligand to said test vesicle;

determining a base amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength;

adding a molecule to said test vesicle;

determining amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission greater than said base amount of fluorescence emission indicates that said molecule has primary ligand-binding activity.

26. The method of claim 25 wherein said primary ligand is calmodulin.

27. The method of claim 25 wherein said purifying comprises affinity chromatography.

28. The method of claim 25 wherein said test vesicle is a test tube or a test well.

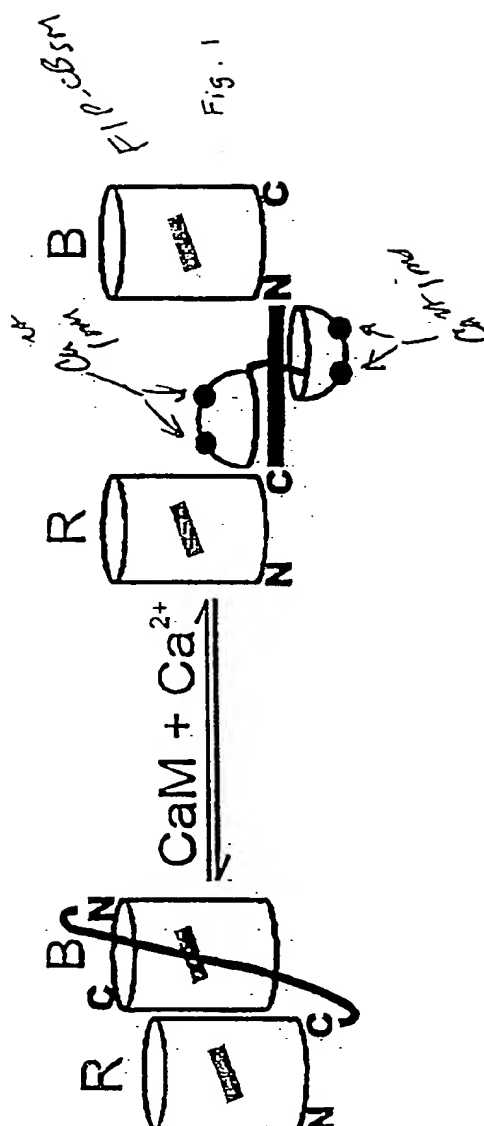


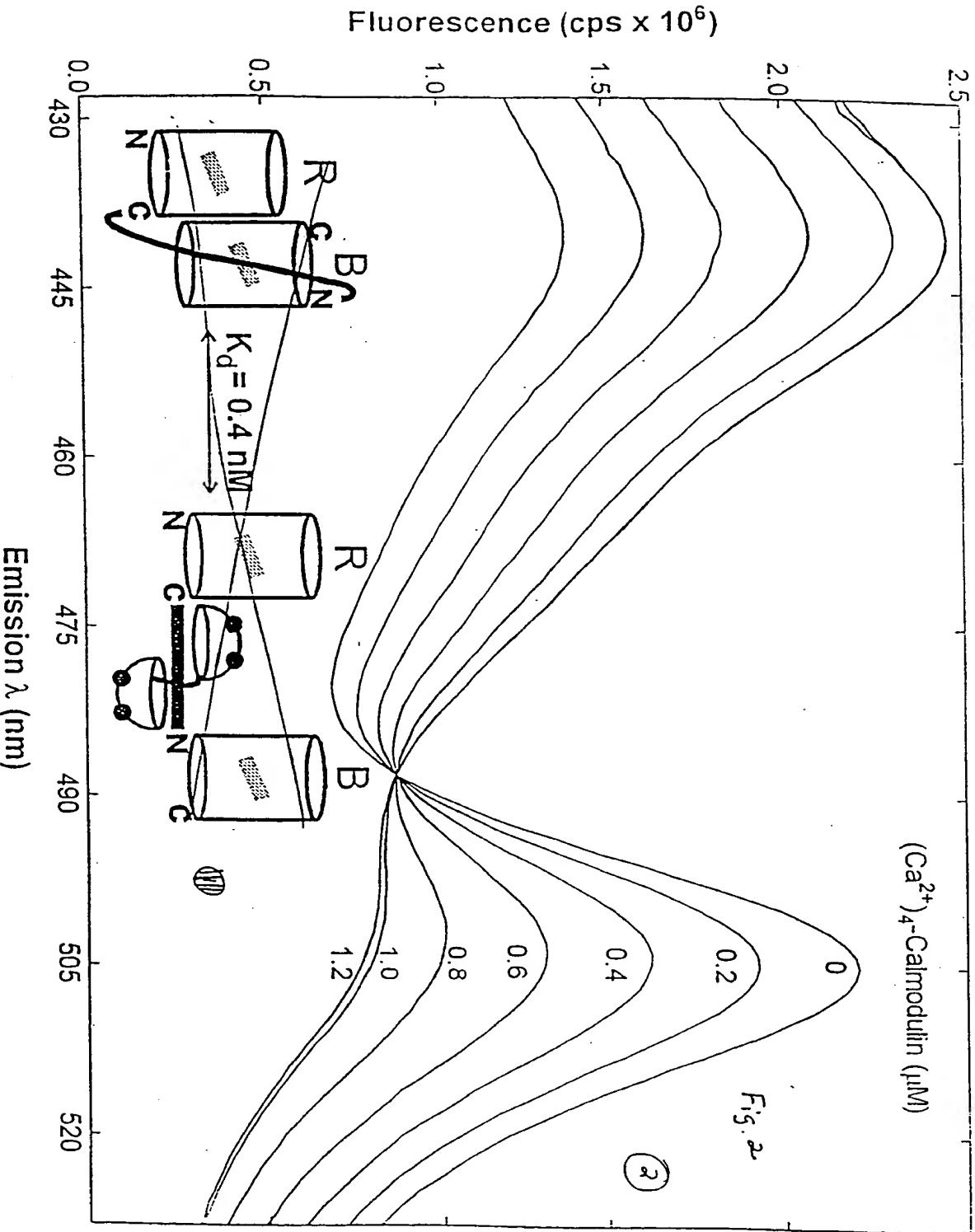
29. A green fluorescent protein complex comprising:  
a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;  
a primary ligand-binding peptide which reversibly binds a primary ligand, said primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and  
a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein,  
wherein when said green fluorescent protein complex is excited at said first wavelength, the amount of fluorescence emission at said third wavelength is reduced when said primary ligand-binding peptide is bound to primary ligand as compared to the amount of fluorescence emission at said third wavelength when said primary ligand-binding peptide is not bound to said primary ligand.
30. The green fluorescent protein complex of claim 29 wherein said primary ligand is calmodulin and said primary ligand-binding peptide is a calmodulin-binding peptide.
31. The green fluorescent protein complex of claim 30 wherein said calmodulin-binding peptide has an amino acid sequence as shown in SEQ ID NO:1.
32. The green fluorescent protein complex of claim 29 wherein said first green fluorescent protein is a blue-shifted green fluorescent protein.
33. The green fluorescent protein complex of claim 32 wherein said blue-shifted green fluorescent protein is excited at a first wavelength maxima of 380 nm and emits fluorescence at a second wavelength maxima of 440 nm.
34. The green fluorescent protein complex of claim 29 wherein said second green fluorescent protein is a red-shifted green fluorescent protein.

35. The green fluorescent protein complex of claim 34 wherein said red-shifted green fluorescent protein is excited at said second wavelength maxima of 495 nm and emits fluorescence at a third wavelength maxima of 505 nm.

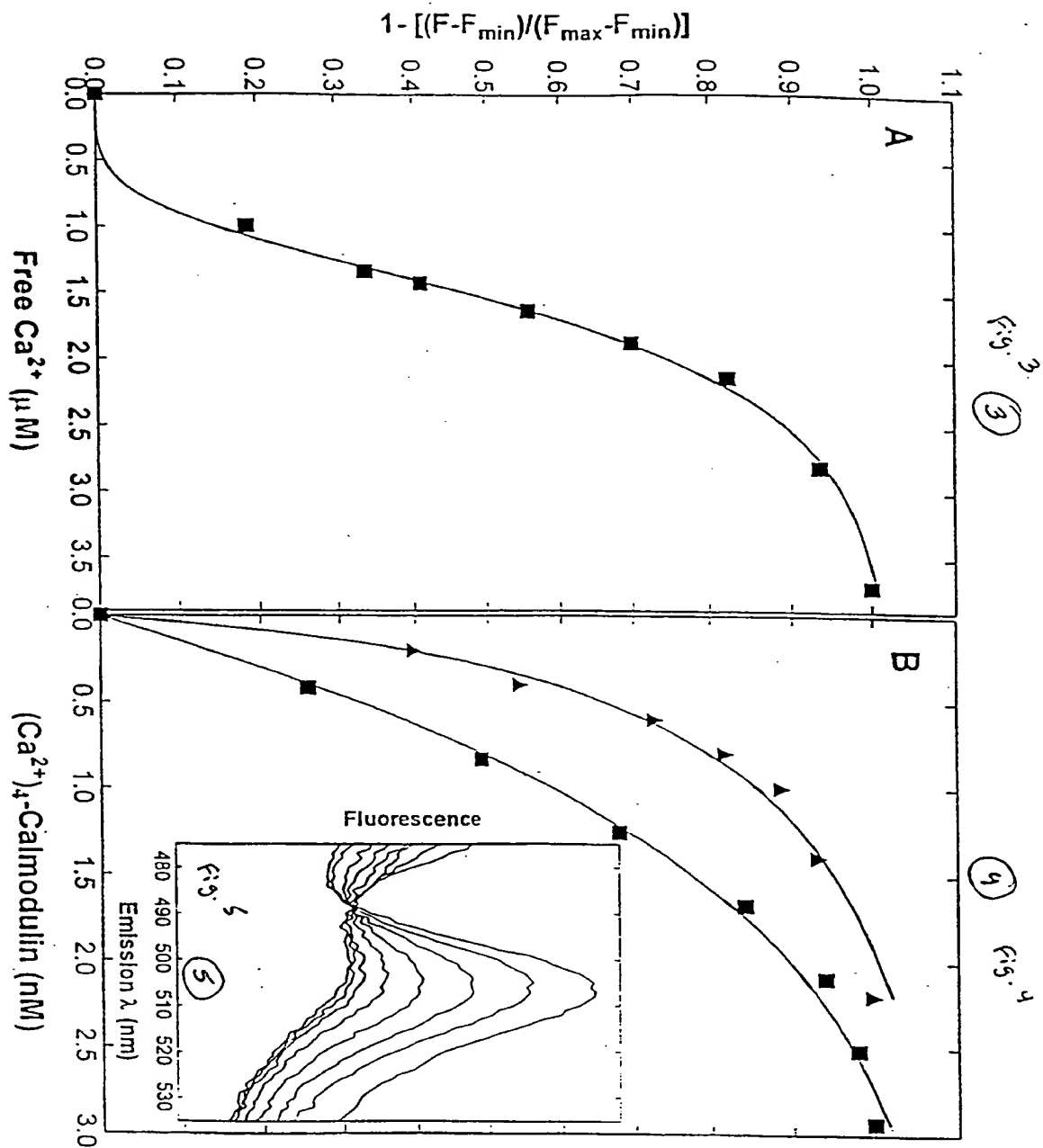
36. The green fluorescent protein complex of claim 29 further comprising said primary ligand covalently attached to one of said first green fluorescent protein or said second green fluorescent protein.

37. A cell comprising the green fluorescent protein complex of claim 29.

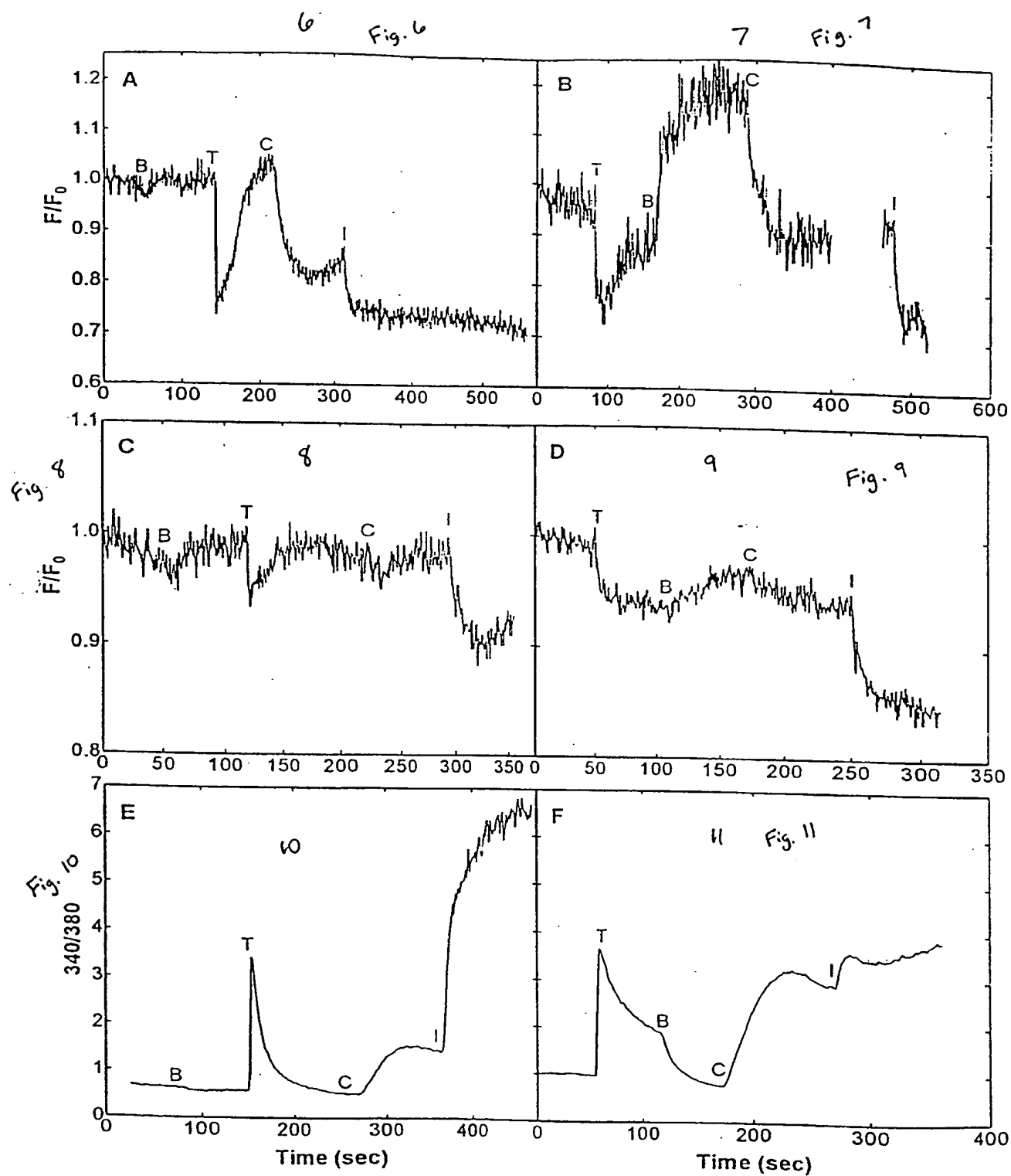




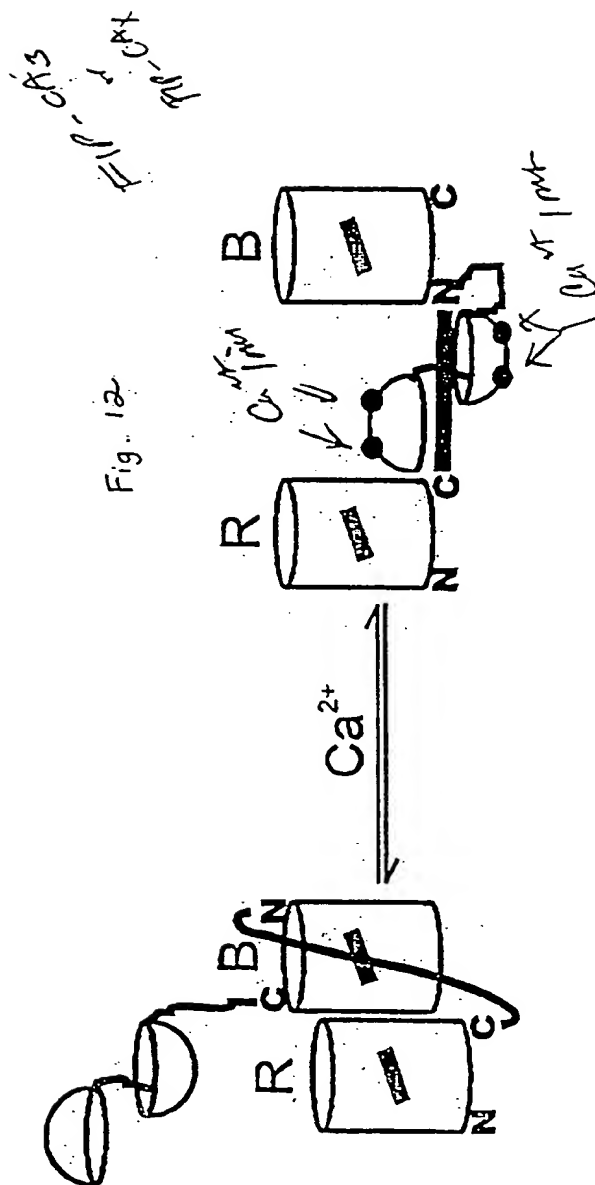
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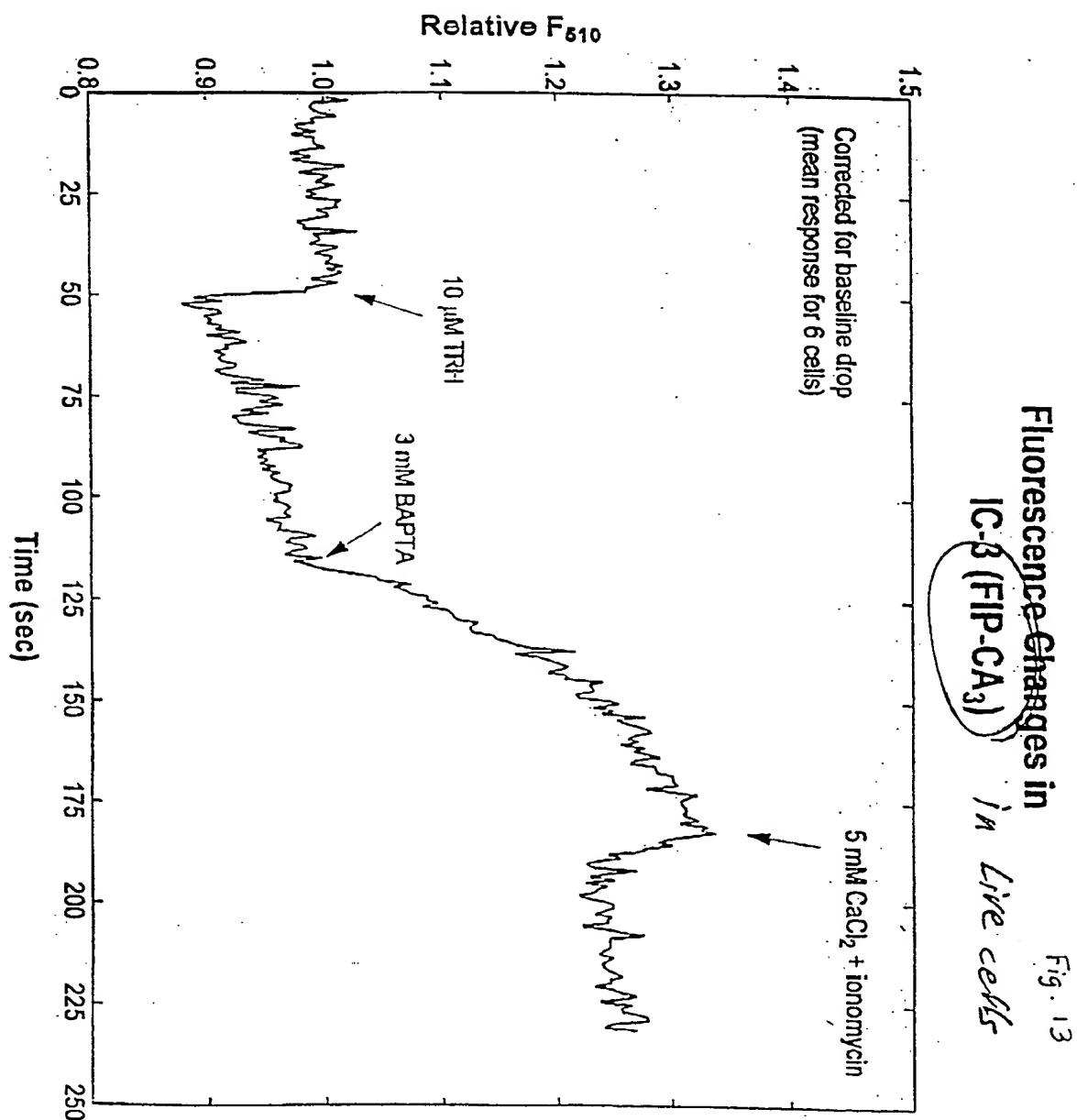
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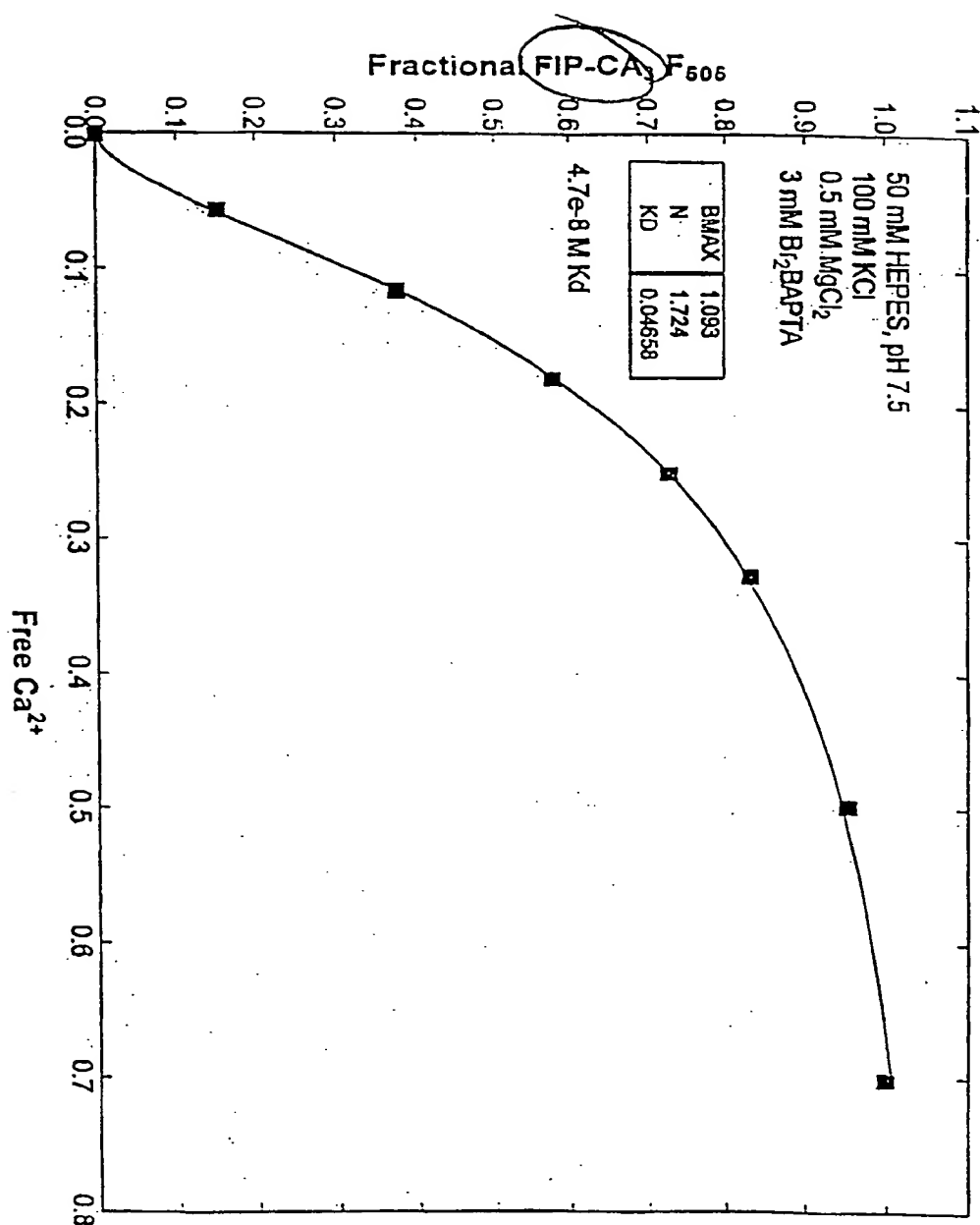


Fig. 14

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08109

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/58 G01N33/533 C12N15/65 C07K14/435 G01N33/542

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CHEMICAL ABSTRACTS, vol. 127, no. 5, 4 August 1997 Columbus, Ohio, US; abstract no. 62698, XP002074734 see abstract & V.A. ROMOSER ET AL.: "Detection in living cells of Ca <sup>2+</sup> -dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 20, 16 May 1997, pages 13270-13274, Bethesda MD USA  --- -/--	1-37

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

17 August 1998

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/08109

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHEMICAL ABSTRACTS, vol. 127, no. 23, 8 December 1997 Columbus, Ohio, US; abstract no. 316403, XP002074735 see abstract & A. PERSECHINI ET AL.: "Novel fluorescent indicator proteins for monitoring free intracellular Ca <sup>2+</sup> ." CELL CALCIUM, vol. 22, no. 3, 2 September 1997, pages 209-216, london uk	1-37
A	WO 96 23898 A (NOVO NORDISK A/S) 8 August 1996 see page 2, line 15 - line 16	1-37
A	US 5 491 084 A (M. CHALFIE ET AL.) 13 February 1996 cited in the application see the whole document	1-37

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 98/08109

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9623898 A	08-08-1996	AU 4483096 A CA 2217700 A EP 0815257 A	21-08-1996 08-08-1996 07-01-1998
US 5491084 A	13-02-1996	AU 7795794 A CA 2169298 A EP 0759170 A JP 9505981 T WO 9507463 A	27-03-1995 16-03-1995 26-02-1997 17-06-1997 16-03-1995



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/58, 33/533, C12N 15/65, C07K 14/435, G01N 33/542</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/48278</b> <b>(43) International Publication Date:</b> 29 October 1998 (29.10.98)
<b>(21) International Application Number:</b> PCT/US98/08109 <b>(22) International Filing Date:</b> 23 April 1998 (23.04.98)  <b>(30) Priority Data:</b> 08/842,322 24 April 1997 (24.04.97) US  <b>(71) Applicant:</b> UNIVERSITY OF ROCHESTER [US/US]; Office of Technology Transfer, 518 Hylan Building, Rochester, NY 14627 (US).  <b>(72) Inventor:</b> PERSECHINI, Anthony; 6 Sunnysbrook Lane, Fairport, NY 14450 (US).  <b>(74) Agents:</b> GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW. ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> DETECTION OF INTRACELLULAR LIGAND BINDING  <b>(57) Abstract</b>  A method of monitoring the amount of primary or secondary ligand in a cell, using a green fluorescent protein complex and fluorescence resonance energy transfer between two GFP molecules to monitor the primary or secondary ligand, is disclosed. Further disclosed is a method of screening a molecule, such as a peptide, for primary ligand-binding activity, which also uses a green fluorescent protein complex and fluorescence resonance energy transfer between two GFP molecules, to screen the molecule or peptide. A green fluorescent protein complex comprising a first green fluorescent protein, a primary ligand-binding peptide, and a second green fluorescent protein, is also disclosed.		

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## DETECTION OF INTRACELLULAR LIGAND BINDING

5           The subject matter of this application was made with support from the United States Government under grant no. DK44322-03 of the National Institutes of Health.

### FIELD OF THE INVENTION

10           The present invention relates generally to cell monitoring and ligand detection and evaluation, and more particularly to a method of monitoring the level of a primary ligand in a cell or of evaluating a molecule for primary ligand-binding activity, using a green fluorescent protein fusion complex that exhibits ligand-dependent fluorescence resonance energy transfer.

### BACKGROUND OF THE INVENTION

15           Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

20           The activity of a cell is influenced and regulated by many molecules. Many cells have receptors which activate pathways within the cell, such as the cAMP pathway or the phosphoinositide pathway. Such receptors are often activated by a ligand. When the ligand binds to its receptor, the receptor activates the pathway. Other molecules influential in a cell include ions such as calcium ions. In an attempt to understand the  
25           molecular biology of a cell, the ways in which cells interact, or even causes of cell death, more knowledge is continually needed regarding the distribution and/or pattern of expression of various molecules within a cell or within a tissue or organ.

          One example of such a molecule is calmodulin. Calmodulin modulates the activities of a large and constantly increasing number of known target proteins (Bredt  
30           and Snyder 1990; Cho et al. 1992; Edelman et al. 1996; Crivici and Ikura 1995; Kink et al. 1990; Lu and Means 1993; Manalan and Klee 1984; Means et al. 1991; Wang et al. 1996). Through their actions it participates in the regulation of most processes in the cell including motility, axonal transport, synaptic transmission, ion homeostasis, cell cycle progression, gene expression and apoptosis. It is of interest to determine whether

maximal and resting free  $(\text{Ca}^{2+})_4$ -calmodulin levels vary among cell types, or at different points in the cell cycle, which has been shown to be associated with cyclic changes in total calmodulin levels. It would also be of interest to investigate spatio-temporal changes in calmodulin activity during cellular events that involve it.

5 It would also be of interest to determine maximal and resting levels of other molecules and ligands, in various cell types, or at different points in the cell cycle, and well as to determine spatio-temporal changes in ligand-binding activity during cellular events that involve it.

## 10 SUMMARY OF INVENTION

To this end, the subject invention provides a method of monitoring the amount of a primary or secondary ligand in a cell. The method first involves the construction of a green fluorescent protein (GFP) complex. The green fluorescent protein complex comprises a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. The constructed green fluorescent protein complex is then introduced into a cell, and a base amount of fluorescence emission at the third wavelength when the cell is excited at the first wavelength is determined. This determination provides a control or base amount of fluorescence resonance energy transfer between the two green fluorescent proteins of the green fluorescent protein complex. The amount of fluorescence emission over time at the third wavelength when the cell is excited over time at the first wavelength is determined. Next, a comparison is made of the amount of fluorescence emission over time to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates an increase in the amount of the primary ligand in the cell. In particular, the primary ligand present in the cell has bound to the primary ligand-binding peptide, changing the distance between the two GFP molecules and altering fluorescence resonance energy transfer (FRET) between them. Changes in



the amount of fluorescence emission over time can be used to monitor changes in the amount of the primary ligand in the cell over time.

By quantitating the amount of fluorescence emission over time using the subject method, one can estimate the amount of the primary ligand in the cell over time.

5       The subject invention further provides a method for monitoring the amount of a secondary ligand in the cell, wherein the secondary ligand binds to the primary ligand and is necessary for binding of the primary ligand to the primary ligand-binding peptide. This method is as recited above, except the method further comprises introducing a known concentration of the primary ligand into the cell prior to determining the base  
10       amount of fluorescence emission. When the amount of fluorescence emission is less than the base amount of fluorescence emission, it is an indication of the presence of the secondary ligand. As above, changes in the amount of fluorescence emission over time indicate changes in the amount of the secondary ligand in the cell over time.

      The invention further provides a method of screening a peptide for primary  
15       ligand-binding activity. The method comprises constructing a green fluorescent protein complex. In this embodiment, the green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein as described above, but they are covalently attached by a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to  
20       the first green fluorescent protein and the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell in the absence of the primary ligand, and a determination of the base amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. The candidate primary ligand is  
25       then added to the cell, and a determination of the amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the molecule has primary ligand-binding activity.  
30       Alternatively, this method of screening a peptide for primary ligand-binding activity can be performed in vitro in, for example, a test tube or well. The green fluorescent protein complex is expressed in a cell and then purified from the cell by conventional protein purification techniques (such as affinity chromatography). The purified protein complex

is placed in a test tube or well and a determination is made of the base amount of fluorescence emission at the third wavelength when the purified protein complex is excited at the first wavelength. The candidate primary ligand is then added to the test tube or well, and a determination is made of the amount of fluorescence emission at the third wavelength when the purified protein complex (in the presence of purified primary ligand) is excited at the first wavelength. As in the previous embodiment, a comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the peptide has primary ligand-binding activity.

Further provided is another method of screening a molecule for the ability to bind a primary ligand in competition with the primary ligand-binding peptide where the constructed green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein covalently attached by a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end. The amino terminal end of the primary ligand-binding peptide is covalently attached to the first green fluorescent protein and the carboxy terminal end of the primary ligand-binding peptide is covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell or test vesicle (such as a test tube or test well) with the primary ligand, and a determination is made of the base amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle are excited at the first wavelength. A molecule, such as a peptide, is then added to the cell or test vesicle, and a determination is made of the amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission greater than the base amount of fluorescence emission indicates that the molecule has primary ligand-binding activity. In particular, the molecule has bound the primary ligand thereby preventing the primary ligand from interfering with energy transfer between the two GFPs (the primary ligand does this by binding to the primary ligand-binding peptide that covalently attaches the two GFPs).

The invention further provides a green fluorescent protein complex which comprises a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide

which reversibly binds a primary ligand, the primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at the second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. When the green fluorescent protein complex is excited at the first wavelength, the amount of fluorescence emission at the third wavelength is reduced when the primary ligand-binding peptide is bound to primary ligand as compared to the amount of fluorescence emission at the third wavelength when the primary ligand-binding peptide is not bound to the primary ligand. Also provided is a cell which expresses the green fluorescent protein complex.

#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 illustrates the conformational change undergone by FIP-CB<sub>SM</sub> upon binding (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin;

Fig. 2 illustrates the calmodulin-dependent changes in the FIP-CB<sub>SM</sub> fluorescence emission spectrum;

Fig. 3 characterizes the calmodulin binding by FIP-CB<sub>SM</sub>, showing the titration of a mixture of 8.2 nM FIP-CB<sub>SM</sub> and 200 nM calmodulin with increasing concentrations of free Ca<sup>2+</sup> ion;

Fig. 4 characterizes the calmodulin binding by FIP-CB<sub>SM</sub>, showing the binding of (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin to FIP-CB<sub>SM</sub> at concentrations of 1 (■) and 1.4 (▲) nM;

Fig. 5 shows the emission spectra for titration of FIP-CB<sub>SM</sub> at a 1.4 nM concentration;

Figs. 6-11 show time courses for area-normalized enhanced fluorescence in cells microinjected with FIP-CB<sub>SM</sub>;

Fig. 12 illustrates the conformational change undergone by FIP-CA<sub>3</sub> upon binding Ca<sup>2+</sup>;

Fig. 13 shows a time course for area-normalized enhanced fluorescence in cells microinjected with FIP-CA<sub>3</sub>; and

Fig. 14 characterizes the calcium binding by FIP-CA<sub>3</sub>, showing the titration of FIP-CA<sub>3</sub> with increasing concentrations of free Ca<sup>2+</sup> ion.

5

### DETAILED DESCRIPTION

The subject invention provides a method of monitoring the amount of a primary or secondary ligand in a cell. The method first involves the construction of a green fluorescent protein complex. The green fluorescent protein complex comprises a first  
10 green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at the second wavelength and  
15 which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. The constructed green fluorescent protein complex is then introduced into a cell and a base amount of fluorescence emission at the third wavelength when the cell is excited at the first wavelength is determined. This determination provides a control or  
20 base amount of fluorescence resonance energy transfer between the two green fluorescent proteins of the green fluorescent protein complex. The amount of fluorescence emission over time at the third wavelength when the cell is excited over time at the first wavelength is determined. Next, a comparison is made of the amount of fluorescence emission over time to the base amount of fluorescence emission, wherein  
25 an amount of fluorescence emission less than the base amount of fluorescence emission indicates an increase in the amount of the primary ligand in the cell. Changes in the amount of fluorescence emission over time can be used to monitor changes in the amount of the primary ligand in the cell over time.

By quantitating the amount of fluorescence emission over time using the subject  
30 method, one can estimate the amount of the primary ligand in the cell over time.

As used herein, a primary ligand refers to any molecule binding directly to the primary ligand-binding peptide in the GFP complex. An example of a primary ligand in accordance with the subject invention is calmodulin (where the primary ligand-binding

peptide is a calmodulin-binding peptide). Various calmodulin-binding peptides are known in the art and each can be used to tether the two green fluorescent proteins together. One calmodulin-binding peptide has the amino acid sequence as shown in SEQ ID NO:1. Examples of additional calmodulin-binding peptides are shown in Table 1 (see Crivici and Ikura 1995, and Persechini et al. 1996).

As further used herein, a green fluorescent protein refers to any fluorescent protein containing a naturally occurring fluorophore. The only requirement is that the two selected green fluorescent proteins have different excitation and fluorescence emission wavelengths. This is necessary to evaluate the fluorescence resonance energy transfer between the two proteins. Two examples of green fluorescent proteins which can be used in accordance with the subject invention are the red-shifted green fluorescent protein (which is excited at a wavelength maxima of 495 nm and emits fluorescence at a wavelength maxima of 505 nm) and the blue-shifted green fluorescent protein (which is excited at a wavelength maxima of 380 nm and emits fluorescence at a wavelength maxima of 440 nm). Other potentially suitable green fluorescent proteins include the GFP from *Aequorea victoria* (U.S. Patent No. 5,491,084;). A plasmid encoding the GFP of *Aequorea victoria* is available from the ATCC as Accession No. 75547. A mutated form of this GFP (a red-shifted mutant form) designated pRSGFP-C1 is commercially available from Clontech Laboratories, Inc. (Palo Alto, California). For further discussions of various GFP molecules, see Crameri et al. 1996; Delegrave et al. 1995; Gura 1996; Inouye and Tsuji 1994; Marshall et al. 1995; Ormo et al. 1996; Yang et al. 1996; and Youvan and Michel-Beyerle 1996.

In the case of the primary ligand calmodulin, as with any other appropriate primary ligand, the binding of calmodulin by the calmodulin-binding peptide may be dependent on the presence of a secondary ligand which binds to the primary ligand. For example, calmodulin requires the presence of calcium ion in order to bind to the calmodulin-binding peptide having SEQ ID NO:1. In these cases, the method of the subject invention further comprises introducing into the cell the secondary ligand necessary for binding of the primary ligand to the primary ligand-binding peptide. The amount of the secondary ligand in the cell can thereby be monitored by introducing the primary ligand into the cell prior to determining the base amount of fluorescence emission. When the amount of fluorescence emission is less than the base amount of fluorescence emission, it is an indication of the presence of the secondary ligand in the

cell. Furthermore, changes in the amount of fluorescence emission over time indicate changes in the amount of the secondary ligand in the cell over time. One embodiment of the subject invention thus provides for the monitoring of such secondary ligands within cells. This is very advantageous, especially for the monitoring of secondary  
5 ligands such as the calcium ion, because the level of calcium ion in a cell can be quantitated by indirect detection via the calcium ion's interaction with calmodulin and the calmodulin-binding peptide. In this embodiment, the primary ligand can be introduced into the cell by covalently attaching the primary ligand to one of the first green fluorescent protein or the second green fluorescent protein in the green fluorescent  
10 protein complex. This fixes the concentration of the primary ligand in relation to the indicator.

Other examples of secondary ligands in accordance with the subject invention include metal ions.

As used herein, covalent attachment refers to a non-reversible attachment  
15 whereas "bound" or "binding" refer to reversible attachments.

The invention further provides a method of screening a peptide for primary ligand-binding activity. The method comprises constructing a green fluorescent protein complex. In this embodiment, the green fluorescent protein complex comprises the first green fluorescent protein and the second green fluorescent protein as described above,  
20 but they are covalently attached by a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to the first green fluorescent protein and the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein. The green fluorescent protein complex is introduced into a cell in the absence of the primary ligand, and a  
25 determination of the base amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. The primary ligand is then added to the cell, and a determination of the amount of fluorescence emission at the third wavelength is made when the cell is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence  
30 emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the peptide has primary ligand-binding activity. Preferably, the primary ligand is calmodulin. Alternatively, this method of screening a peptide for primary ligand-binding activity can be performed in vitro in, for example, a

test tube or well. The green fluorescent protein complex is expressed in a cell and then purified from the cell by conventional protein purification techniques (such as affinity chromatography). The purified protein complex is placed in a test tube or well and a determination is made of the base amount of fluorescence emission at the third  
5 wavelength when the purified protein complex is excited at the first wavelength. Purified primary ligand is then added to the test tube or well, and a determination is made of the amount of fluorescence emission at the third wavelength when the purified protein complex (in the presence of purified primary ligand) is excited at the first wavelength. As in the previous embodiment, a comparison is then made of the amount  
10 of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission less than the base amount of fluorescence emission indicates that the peptide has primary ligand-binding activity.

Further provided is a method of screening a molecule for the ability to bind a known primary ligand where the constructed green fluorescent protein complex  
15 comprises the first green fluorescent protein and the second green fluorescent protein covalently attached by a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end. The amino terminal end of the primary ligand-binding peptide is covalently attached to the first green fluorescent protein and the carboxy terminal end of the primary ligand-binding peptide is covalently attached to the second  
20 green fluorescent protein. The green fluorescent protein complex is introduced into a cell or test vesicle with the primary ligand, and a determination is made of the base amount of fluorescence emission at the third wavelength when the cell or contents of the test vesicle is excited at the first wavelength. A molecule, such as a peptide, is then added to the cell or test vesicle, and a determination is made of the amount of  
25 fluorescence emission at the third wavelength when the cell or contents of the test vesicle is excited at the first wavelength. A comparison is then made of the amount of fluorescence emission to the base amount of fluorescence emission, wherein an amount of fluorescence emission greater than the base amount of fluorescence emission indicates that the molecule has primary ligand-binding activity. As above, preferably  
30 the primary ligand is calmodulin and the primary ligand-binding peptide (a calmodulin-binding peptide) has an amino acid sequence as shown in SEQ ID NO:1. This method of screening a molecule for the ability to bind a known primary ligand can also be

performed in vitro in, for example, a test tube or well, as discussed above for the in vitro screening of a peptide for the ability to bind a known primary ligand.

The invention further provides a green fluorescent protein complex which comprises a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength; a primary ligand-binding peptide which reversibly binds a primary ligand, the primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and a second green fluorescent protein which is excited at the second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein. When the green fluorescent protein complex is excited at the first wavelength, the amount of fluorescence emission at the third wavelength is reduced when the primary ligand-binding peptide is bound to primary ligand as compared to the amount of fluorescence emission at the third wavelength when the primary ligand-binding peptide is not bound to the primary ligand. Also provided is a cell which expresses the green fluorescent protein complex. Suitable cells include bacterial and mammalian cells, including *Escherichia coli* cells.

Preferably, the primary ligand is calmodulin and the primary ligand-binding peptide is a calmodulin-binding peptide, such as the peptide having the amino acid sequence as shown in SEQ ID NO:1. The first green fluorescent protein is preferably a blue-shifted green fluorescent protein (which is excited at a first wavelength maxima of 380 nm and emits fluorescence at a second wavelength maxima of 440 nm and emits the second green fluorescent protein is preferably a red-shifted green fluorescent protein (which is excited at the second wavelength maxima of 495 nm and emits fluorescence at a third wavelength maxima of 505 nm). In one embodiment, the green fluorescent protein complex may further comprise the primary ligand covalently attached to one of the first green fluorescent protein or the second green fluorescent protein so as to provide for detection of a second ligand. Here the sensitivity of the indicator to the secondary ligand can be modified by altering the interface between the primary ligand and the primary ligand-binding peptide.

Standard techniques can be used to replace the GFPs of plasmid pFIP-CB<sub>SM</sub> with alternative GFPs, and standard techniques can be used to replace the calmodulin-binding



peptide of plasmid pFIP-CB<sub>SM</sub> with alternative primary ligand-binding peptides (or a peptide to be screened for primary ligand-binding activity). Generally, this involves the use of restriction enzymes and ligation (see below).

The green fluorescent protein complex according to the subject invention can be introduced into a cell. Techniques for introducing the complex or a deoxyribonucleic acid molecule in the form of a plasmid allowing expression of the complex into a cell are known in the art. These include: 1) microinjection, in which the purified complex or RNA is injected directly into the cell through fine glass needles; 2) dextran incubation, in which DNA is incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell; 3) calcium phosphate coprecipitation, in which cells efficiently take in DNA in the form of a precipitate with calcium phosphate; 4) electroporation, in which cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage through these vesicles may sometimes destroy or damage DNA); 5) liposomal mediated transformation, in which DNA is incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm; 6) biolistic transformation, in which DNA is absorbed to the surface of gold particles and fired into cells under high pressure using a ballistic device; and 7) viral-mediated transformation, in which nucleic acid molecules are introduced into cells using viral vectors. Since viral growth depends on the ability to get the viral genome into cells, viruses have devised efficient methods for doing so. These viruses include retroviruses and lentivirus, adenovirus, herpesvirus, and adeno-associated virus. Preferably, the plasmid is introduced into a cell by microinjection or the use of a vector (a plasmid or viral vector, for example). U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase.

These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

5       The methods of the subject invention use fluorescence resonance energy transfer (FRET) between spectral variants of GFP to monitor primary ligands or secondary ligands. FRET is described in more detail in Mitra et al. 1996. Briefly, FRET is a process in which an excited fluorophore (the donor) transfers its excited state energy to a light absorbing molecule (the acceptor). Generally, the fluorescence of the donor  
10       decreases as the fluorescence of the acceptor increases and vice versa.

### EXAMPLE I

      The  $\text{Ca}^{2+}$ -binding protein, calmodulin, is a key transducer of intracellular  $\text{Ca}^{2+}$  ion signals, mainly through  $\text{Ca}^{2+}$ -dependent modulation of numerous enzyme activities  
15       (Bredt and Snyder 1990; Cho et al. 1992; Edelman et al. 1996; Crivici and Ikura 1995; Kink et al. 1990; Lu and Means 1993; Manalan and Klee 1984; Means et al. 1991; Wang et al. 1996). This example describes a fluorescent indicator protein whose emission changes reversibly from green to blue light when it binds  $(\text{Ca}^{2+})_4$ -calmodulin ( $K_d=0.4$  nM), in analogy with a calmodulin-dependent enzyme activity. This response  
20       has been monitored in cells microinjected with the indicator. It closely mirrors changes in the intracellular free  $\text{Ca}^{2+}$  ion concentration, responding to a rapid, receptor-mediated, increase with no discernable lag (<300 ms). The fractional indicator response that can be achieved in the absence of co-injected calmodulin is consistent with a free intracellular  $(\text{Ca}^{2+})_4$ -calmodulin concentration of ~1 nM. Images using green/blue  
25       fluorescence ratios demonstrate the utility of this indicator for investigating spatio-temporal changes in  $(\text{Ca}^{2+})_4$ -calmodulin levels in living cells.

      The fluorescent indicator protein (FIP- $\text{CB}_{\text{SM}}$ ) contains two previously characterized green fluorescent protein (GFP) variants; one with fluorescence excitation and emission maxima of ~380 and ~440 nm (BGFP; Heim et al. 1994), the other with  
30       excitation and emission maxima of ~495 and ~505 nm (RGFP; Delegrave et al. 1995). These are joined by a linker sequence containing the calmodulin-binding domain of avian smooth muscle myosin light chain kinase (Fig. 1). When excited at 380 nm, FIP- $\text{CB}_{\text{SM}}$  exhibits a pronounced emission peak at 505 nm, which is reduced by 65% when

Ca<sup>2+</sup>-calmodulin is bound to the linker. There is a corresponding increase in F<sub>440</sub> so that the F<sub>440</sub>/F<sub>505</sub> emission ratio increases from a value of 0.6 to a value of 3 when FIP-CB<sub>SM</sub> is fully saturated with calmodulin. This behavior is consistent with a calmodulin-dependent reduction in FRET between the fluorophores in the two GFP domains in FIP-CB<sub>SM</sub> (Fig. 2). This is the first example of a fusion between two GFPs that exhibits reversible changes in inter-fluorophore FRET in response to a ligand. The fluorescence properties of FIP-CB<sub>SM</sub> make it suitable for single-wavelength and emission-ratio measurements. FIP-CB<sub>SM</sub> binds (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin with a K<sub>d</sub> of 0.4 nM, which is close to the 1 nM apparent value inferred for the complex between calmodulin and smooth muscle myosin light chain kinase (Figs. 3 and 4) (Adelstein et al. 1981).

Purified GFP is known to dimerize in solution, and crystallographic data suggest that the two subunits are in an antiparallel orientation, placing the N-terminus of one protein about 70 Å from the C-terminus of the other (Ormo et al. 1996; Yang et al. 1996). Modeling studies suggest that the linker in FIP-CB<sub>SM</sub> should allow the two GFP domains to associate similarly. The modest ionic strength dependence of the FIP-CB<sub>SM</sub> emission spectrum measured in the absence of calmodulin may reflect the presence of stabilizing electrostatic interactions between amino acids at the interface between the two GFP domains. Perhaps because of changes in the orientation between the two GFP domains, the efficiency of FRET between the fluorophores in FIP-CB<sub>SM</sub> appears to depend upon the length of the linker sequence. The F<sub>440</sub>/F<sub>505</sub> ratio increases from a value of 0.6 with the 26 amino acid linker in FIP-CB<sub>SM</sub>, to 0.9 and 1.5 with 20 and 11 amino acid linkers, respectively. As seen in the crystal structure, the two chromophores in a GFP dimer are ~25 Å apart (Yang et al. 1996). The complex between (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin and the smooth muscle myosin light chain kinase calmodulin-binding domain forms a globular structure 40 Å in diameter (Crivici and Ikura 1995). When calmodulin binds to the kinase calmodulin-binding domain in the FIP-CB<sub>SM</sub> linker, it adopts an α-helical conformation. In this conformation the 17 residue sequence has a length of ~30 Å, about half its length in an extended conformation. Thus, when (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin is bound to the linker in FIP-CB<sub>SM</sub>, the distance between the fluorophores is likely to increase from ~25 Å to ~65 Å, with calmodulin tightly sandwiched between the two GFP domains (Fig. 1). The efficiency of FRET depends upon the distance between the donor and acceptor fluorophores and upon the relative orientation of their dipole moments (Stryer 1978). Changes in the distance and possibly also the orientation

parameters is clearly the basis for the observed calmodulin-dependent change in the FIP-CB<sub>SM</sub> fluorescence emission spectrum. In attempting to design FIPs with novel specificities, it is important to bear in mind that shortening of the linker sequence in FIP-CB<sub>SM</sub> when (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin is bound may paradoxically help to force the GFP domains apart.

Purified FIP-CB<sub>SM</sub> has been microinjected into HEK-293 cells stably transfected with epitope-tagged thyrotropin releasing hormone (TRH) receptor, a Ca<sup>2+</sup>-mobilizing G-protein-coupled receptor (Nelson and Hinkle 1994). The response of the FIP-CB<sub>SM</sub> in these cells to externally applied TRH, ionomycin and 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was measured. The concentration of the FIP-CB<sub>SM</sub> solution used in microinjection experiments was 80 μM, and an estimate of intracellular FIP-CB<sub>SM</sub> is in a concentration range of 1-10 μM in microinjected cells, similar to estimates for the intracellular concentrations of high-abundance calmodulin targets, including smooth muscle myosin light chain kinase, calcineurin, calmodulin-dependent protein kinase II and cerebellar nitric oxide synthase (Tansey et al. 1994 ). When FIP-CB<sub>SM</sub> fluorescence is excited at 380 nm, images of whole cells using an F<sub>510</sub>/F<sub>440</sub> emission ratio and time courses for relative changes in whole-cell F<sub>510</sub> exhibit striking responses to changes in the intracellular Ca<sup>2+</sup> ion concentration (Figs. 6-11). The results suggest no discernable lag (<300 ms) between activation of TRH receptor and formation of significant levels of (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin-target complexes in the nucleus or cytoplasm (Figs. 6-11).

To estimate the maximal intracellular FIP-CB<sub>SM</sub> response, cells were injected with a 1:1 mole ratio of FIP-CB<sub>SM</sub> and calmodulin. In spite of the higher and more sustained Ca<sup>2+</sup> levels caused by ionomycin treatment, both ionomycin and TRH elicit a similar FIP-CB<sub>SM</sub> response in these cells, suggesting that is near the maximum (Figs. 6 and 7). The average decrease in F<sub>510</sub> caused by ionomycin or TRH is ~30%. The average ionomycin-dependent decrease in F<sub>510</sub> seen in cells injected with FIP-CB<sub>SM</sub> alone is ~10% (Figs. 7 and 8). This difference between cells injected with FIP-CB<sub>SM</sub> alone and those injected with FIP-CB<sub>SM</sub>/calmodulin appears to be independent of the intracellular FIP-CB<sub>SM</sub> concentration as estimated from the area-normalized F<sub>510</sub> of resting cells, which varies by a factor of 8 among different cells. The lower FIP-CB<sub>SM</sub> response exhibited in cells injected with the indicator alone suggests a limiting free (Ca<sup>2+</sup>)<sub>4</sub>-calmodulin concentration in the cell. This is also consistent with the

significantly greater reduction in  $F_{510}$  caused by ionomycin treatment of these cells, compared with that caused by TRH treatment, since the higher and more sustained  $\text{Ca}^{2+}$  ion levels resulting from ionomycin treatment would be expected to lead to higher levels of  $(\text{Ca}^{2+})_4$ -calmodulin (Figs. 6-11).

5        Assuming that the  $F_{510}$  value measured in the presence of BAPTA represents completely unliganded FIP- $\text{CB}_{\text{SM}}$ , and that  $F_{510}$  value measured in ionomycin treated cells injected with FIP- $\text{CB}_{\text{SM}}$ /calmodulin represents fully liganded FIP- $\text{CB}_{\text{SM}}$ , then the fractional response to ionomycin seen with cells injected only with FIP- $\text{CB}_{\text{SM}}$  corresponds to a free  $(\text{Ca}^{2+})_4$ -calmodulin level of 0.2 nM. Treatment with BAPTA can  
10        cause  $F_{510}$  in cells injected with FIP- $\text{CB}_{\text{SM}}$  or FIP- $\text{CB}_{\text{SM}}$ /calmodulin to increase above the value measured at the start of the experiment (Figs. 10 and 11). This indicates a significant basal level of  $(\text{Ca}^{2+})_4$ -calmodulin, which is unfortunately difficult to quantitate. However, the 30% reduction in  $F_{510}$  measured in cells microinjected with FIP- $\text{CB}_{\text{SM}}$ /calmodulin represents ~50% of the indicator's dynamic range, so the basal  
15         $(\text{Ca}^{2+})_4$ -calmodulin concentration in cells is unlikely to exceed 0.4 nM (Fig. 2). One can therefore conservatively estimate that the average free  $(\text{Ca}^{2+})_4$ -calmodulin level increases to ~1 nM in microinjected cells treated with ionomycin. As with any intracellular indicator, FIP- $\text{CB}_{\text{SM}}$  undoubtedly affects homeostasis of the ligand it is designed to detect:  $(\text{Ca}^{2+})_4$ -calmodulin.

20        A low maximal level of  $(\text{Ca}^{2+})_4$ -calmodulin in cells is consistent with mobility studies of microinjected tagged calmodulin, which indicate that >95% of the total calmodulin is bound to other intracellular proteins at a saturating free  $\text{Ca}^{2+}$  ion concentration. A low maximal level of  $(\text{Ca}^{2+})_4$ -calmodulin also suggests that for a typical target, with a 1 nM dissociation constant for  $(\text{Ca}^{2+})_4$ -calmodulin, changes in  
25        calmodulin-binding affinity will result in proportional changes in calmodulin-dependent target activity. In agreement with this, Stull et al. (1993) have demonstrated that a 10-fold decrease in the affinity of smooth muscle myosin light chain kinase for  $(\text{Ca}^{2+})_4$ -calmodulin significantly reduces levels of this enzyme activity in smooth muscle cells.

      Cells injected cytoplasmically with FIP- $\text{CB}_{\text{SM}}$  exhibit a diffuse fluorescence that  
30        is excluded from the nucleus and other cellular organelles. Imaging using the  $F_{510}/F_{440}$  ratio shows an obvious  $\text{Ca}^{2+}$ -dependent FIP- $\text{CB}_{\text{SM}}$  response in cells injected either with the FIP- $\text{CB}_{\text{SM}}$  alone or with FIP- $\text{CB}_{\text{SM}}$ /calmodulin. A clear FIP- $\text{CB}_{\text{SM}}$  response is observed in both cytoplasm and nucleus. It has been suggested that calmodulin plays an

important role in controlling events in the nucleus, including mitosis (Lu et al. 1993; Wang et al. 1996). The results certainly indicate that comparable levels of free  $(\text{Ca}^{2+})_4$ -calmodulin are achieved in the nucleus and cytosol. There appear to be regional variations in the FIP-CB<sub>SM</sub> response that may represent a heterogeneous distribution of calmodulin and/or free  $\text{Ca}^{2+}$  ion in the cell.

## EXAMPLE II

Fig. 2 shows the calmodulin-dependent changes in the FIP-CB<sub>SM</sub> fluorescence emission spectrum. FIP-CB<sub>SM</sub> at a concentration of 1  $\mu\text{M}$  was titrated with 0.2  $\mu\text{M}$  increments of calmodulin in the presence of 300  $\mu\text{M}$   $\text{CaCl}_2$ . The buffer also contained 25 mM Tris-HCl, pH 7.5 and 0.1 M NaCl. Fluorescence was excited at 380 nm. The effects of calmodulin on the FIP-CB<sub>SM</sub> emission spectra are completely reversed by 5 mM EDTA. A scheme depicting the conformational change undergone by FIP-CB<sub>SM</sub> upon binding  $(\text{Ca}^{2+})_4$ -calmodulin is presented in Fig. 1. The RGFP (R) and BGFP (B) domains in FIP-CB<sub>SM</sub> are joined by the linker sequence: SEQ ID NO:1: **GTSSRRKWNKTGHAVRAIGRLSSTGA**, which contains the calmodulin-binding domain from avian smooth muscle myosin light chain kinase (Guerriero et al. 1986), shown in boldface type. The fluorophores in the GFP domains are represented by shaded rectangles. The shape used for the GFP domains is based upon the published crystal structures for GFP, which indicate an eleven-stranded  $\beta$ -barrel (Ormo et al. 1996; Yang et al. 1996). Based upon the corresponding calmodulin-peptide structure, the linker sequence in FIP-CB<sub>SM</sub> is enfolded by the two lobes of  $(\text{Ca}^{2+})_4$ -calmodulin and adopts an  $\alpha$ -helical conformation. Calmodulin is depicted as two hemispheres, corresponding to the lobes, joined by the flexible central helix tether (Persechini and Kretsinger 1988). The two  $\text{Ca}^{2+}$  ions bound to each lobe in  $(\text{Ca}^{2+})_4$ -calmodulin are depicted as filled circles. FIP-CB<sub>SM</sub> was expressed in *E. coli* and purified essentially as described by Mitra et al. (1996). The purified protein exhibits optical absorbance maxima at 480 and 380 nm with respective  $\epsilon$  values of 89 and 36  $\text{mM}^{-1}\text{cm}^{-1}$ , which correspond with the absorbances of RGFP and BGFP, respectively. The 380 nm-excited fluorescence emission spectrum of a control protein in which the linker sequence used in FIP-CB<sub>SM</sub> is replaced by the sequence: SEQ ID NO:2: GTSSGSSTTGA is unaffected by  $(\text{Ca}^{2+})_4$ -calmodulin levels as high as 3  $\mu\text{M}$ . The FIP-CB<sub>SM</sub> emission spectrum is independent of pH between 7.0 and 8.0, either in the presence or absence of bound

calmodulin. The emission spectrum of FIP-CB<sub>SM</sub> is affected by changes in ionic strength; the  $F_{440}/F_{505}$  emission ratio increases by 0.1 with each 50 mM increment in ionic strength between 130 and 300 mM. In the presence of a saturating level of  $(Ca^{2+})_4$ -calmodulin the  $F_{440}/F_{505}$  ratio of FIP-CB<sub>SM</sub> is unaffected by ionic strength changes in this range.

### EXAMPLE III

Figs. 3-5 show the characterization of calmodulin binding by FIP-CB<sub>SM</sub>. Fig. 3 shows the titration of a mixture of 8.2 nM FIP-CB<sub>SM</sub> and 200 nM calmodulin with increasing concentrations of free  $Ca^{2+}$  ion. Free  $Ca^{2+}$  ion concentrations were established by incremental additions of standard  $CaCl_2$  solutions to a buffer containing 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.5 mM  $MgCl_2$ , and 3 mM 1,2-bis(o-amino-5-5'-dibromophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid ( $Br_2$ BAPTA) at 25°C. Values for the free  $Ca^{2+}$  concentration given in the figure were calculated using the MaxChelator software package (Bers et al. 1994). The curve shown in the figure was calculated using an equation of the form:  $F = \alpha (L^n)/(L^n + K_a)$  where  $F$  is the fractional saturation of FIP-CB<sub>SM</sub> with calmodulin, given in the figure as  $1 - [(F - F_{min})/(F_{max} - F_{min})]$ ,  $L$  is the free  $Ca^{2+}$  ligand concentration,  $K_a$  is an apparent dissociation constant that depends upon the total amount of calmodulin, and  $n$  is the number of interacting sites, which equaled 3.9 for the curve shown. This indicates that  $(Ca^{2+})_4$ -calmodulin is the species bound by FIP-CB<sub>SM</sub>.  $F$  is the fluorescence measured at 505 nm,  $F_{max}$  and  $F_{min}$  are the values for  $F$  measured at maximal and minimal free  $Ca^{2+}$  ion concentrations.  $\alpha$  is a correction factor allowing adjustment of the maximal fraction of FIP-CB<sub>SM</sub> bound to give the best fit.  $\alpha$  values are  $<1.1$  for the curves shown in panels A and B.

Fig. 4 shows the binding of  $(Ca^{2+})_4$ -calmodulin to FIP-CB<sub>SM</sub> at concentrations of 1 (■) and 1.4 (▲) nM. Buffer conditions are described above for Fig. 2 (Example II). The curve fitting data measured at a 1 nM FIP-CB<sub>SM</sub> concentration was generated according to a standard single-site kinetic model. The curve fitting data measured at a 1.4 nM FIP-CB<sub>SM</sub> concentration was generated according to an equation of the form:  $F = \alpha \{ (P_t + L_t + K_d) - [(P_t + L_t + K_d)^2 - 4(P_t)(L_t)]^{0.5} \} / 2(P_t)$ , where  $L_t$  is the total calmodulin concentration and  $P_t$  is the total FIP-CB<sub>SM</sub> concentration. In both cases the curves fitting the data were generated using a  $K_d$  value of 0.4 nM. Emission spectra for titration of FIP-CB<sub>SM</sub> at a 1.4 nM concentration are shown in Fig. 5.

#### EXAMPLE IV

Figs. 6-11 show time courses for area-normalized enhanced fluorescence in cells microinjected with FIP-CB<sub>SM</sub>. FIP-CB<sub>SM</sub> fluorescence was excited at 380 nm and measured at 510 nm. Data are presented as simple fraction of the  $F_{510}$  measured at the start of the experiment ( $F_0$ ), except for fura-2 measurements, which are presented as 340/380 fluorescence excitation ratios, determined as previously described. All traces are averages of data collected from 8-15 cells. B, T, C and I indicate external applications of 3 mM BAPTA, 1  $\mu$ M TRH, 3 mM CaCl<sub>2</sub> and 3  $\mu$ M ionomycin. Figs. 6 and 7 contain traces for cells injected with FIP-CB<sub>SM</sub> and calmodulin in a 1:1 mole ratio. Traces for cells injected with FIP-CB<sub>SM</sub> alone are presented in Figs. 8 and 9. Figs. 10 and 11 contain traces for the fura-2 response of mock-injected cells. It was found that microinjection of a synthetic peptide based on the calmodulin-binding domain in skeletal muscle myosin light chain kinase (Blumenthal and Krebs 1987) at a concentration identical to that used for FIP-CB<sub>SM</sub> has no discernable effect on the fura-2 response. This was also found to be the case for cells microinjected with concentrations of calmodulin comparable to those co-injected with FIP-CB<sub>SM</sub>. Cells microinjected with a control GFP fusion protein (see above description of Fig. 2; Example II), either alone or in a 1:1 mole ratio with calmodulin, exhibited no significant Ca<sup>2+</sup>-dependent changes in  $F_{510}$ . The gap in the trace presented in Fig. 7 is due to a camera malfunction.

#### EXAMPLE V

A second fluorescent indicator protein (FIP-CA<sub>3</sub>) useful for monitoring levels of secondary ligands is shown in Fig. 12. In this embodiment, the calmodulin protein (the primary ligand) is covalently attached to the green fluorescent protein complex described in the above examples. FRET in this embodiment is dependent only on Ca<sup>2+</sup> (a secondary ligand) levels.

Fig. 13 shows the time course for area-normalized enhanced fluorescence in cells microinjected with FIP-CA<sub>3</sub> (comparable to Figs. 6-11 for FIP-CB<sub>SM</sub>).

Fig. 14 characterizes the calcium binding by FIP-CA<sub>3</sub>, showing the titration of FIP-CA<sub>3</sub> with increasing concentrations of free Ca<sup>2+</sup> ion.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the



invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table 1 Primary Sequences of some known and putative calmodulin binding domains of protein and peptide calmodulin targets

Target <sup>a</sup>	Sequence <sup>b</sup> (with SEQ ID NO. in parentheses)	Reference
skMLCK (M13) (c)	K R R W K K K N F I A V S A A N R F K K I S S S G A L (3)	Blumenthal et al. 1987
smMLCK (smMLCKp)	A R R K W Q K T G H A V R A I G R L S S (4)	Lowenstein et al. 1992
CaMKII	A R R K L K G A I L T T M L A T R N F S (5)	Novack et al. 1991
Caldesmon	G V R N I K S M W E K G N V F S S (6)	Zhang et al. 1994
Caldesmon	A R R K L K A A V K A V A S S R L G S (7)	Payne et al. 1988
Caldesmon	F M N N W E V Y K L L A H I R P P A P K S Y T V (8)	Buschmeier et al. 1987
PFK (M11)	A R K E V I R N K L A H I R P P A P K S Y T V (9)	Kincaid et al. 1988
Calcineurin	L R R L I D A Y A F R I Y G H W V K K G Q Q Q N R G (10)	Dasgupta et al. 1989
PhK (PhK5)	R G K F K V I C L T V L A S V R I Y Y Q Y R R V K P G (11)	Dasgupta et al. 1989
PhK (PhK13)	L R R G Q I L W F R G L N R I Q T Q I K V V N A F S S (12)	Vorherr et al. 1990
Ca <sup>2+</sup> -ATPase (C28W)	T E K K H L Q R P I F R L R C L V K Q L E K (13)	Vorherr et al. 1993
59-kDa PDE	K R R A I G E F K K L A E A V L V Q L M H C R K M F K A (16)	Vorherr et al. 1993
60-kDa PDE	K R R A I G E F K K L A E A V L V Q L M H C R K M F K A (17)	Vorherr et al. 1993
NOS (NO-30)	I K P A K R M K F I D L L W K I A R A G A R S A V G T E A (18)	Oldenburger et al. 1992
Type I AC (AC-28)	K A H K A A T K I Q A S F R L M H I T R K K L K G E K K (19)	Chapman et al. 1991
Bordetella pertussis AC	K T A S P W K S A R L M V H T V A T F N S I K E (20)	Leto et al. 1989
Neuromodulin	K K K K R F S F K K P F K L S G L S F K K R N R K (21)	Graff et al. 1991
Spectrin	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (24)	Blackshear et al. 1992
MARCKS	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (25)	Scaramuzzino 1993
F52 or MacMARCKS	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (26)	Minami et al. 1993
β-Adducin	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (27)	Srinivas et al. 1993
HSP90α	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (28)	Mercer et al. 1991
HIV-1 gp160	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (29)	Mercer et al. 1991
BBMHCI	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (30)	Malencik et al. 1983b
Dilute MHC	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (31)	Malencik et al. 1983b
Mastoparan	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (32)	Malencik et al. 1983a
Melittin	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (33)	Malencik et al. 1983a
Glucagon	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (34)	Malencik et al. 1983a
Secretin	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (35)	Malencik et al. 1983a
VIP	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (36)	Malencik et al. 1983a
GIP	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (37)	Malencik et al. 1983a
Model peptide CBP2	K Q K K E K D Q Y H R L R D L L I V K R I V E L L G R R (38)	DeGrado et al. 1985

<sup>a</sup> Abbreviations: AC, adenylyl cyclase; BBMHCI, brush-border myosin heavy chain-I; CaMKII, calmodulin kinase II; CBP2, calmodulin binding peptide-2; GIP, gastrin inhibitory peptide; HIV-1 gp160, human immunodeficiency virus envelope glycoprotein 160; HSP, heat-shock protein; MARCKS, myristoylated alanine-rich C kinase substrate; MHC, myosin heavy chain; NOS, nitric oxide synthase; PDE, phosphodiesterase; PFK, phosphofructokinase; PhK, phosphorase kinase; sk-, smMLCK, skeletal muscle- and smooth muscle-myosin light chain kinase; VIP, vasoactive intestinal peptide.

<sup>b</sup> Alignment of the CaM domains was made by visual inspection based on alignment of the putatively conserved major (bold and underlined) and minor (bold) hydrophobic anchors that interact with the hydrophobic patches of the C- and N-terminal domains of CaM (Ikura et al. 1992), and on the alignment of the conserved basic residue (bold and italicized) analogous to that residue of MLCK that is required for activation by CaM (Meador et al. 1992; Meador et al. 1993). Precise boundaries of the CaM-binding domain are not known for all targets.

<sup>c</sup> Names in parentheses are those used in the literature for the synthetic peptides containing the sequences listed.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: University of Rochester
- (ii) TITLE OF INVENTION: DETECTION BY FRET CHANGES OF  
LIGAND BINDING BY GFP FUSION  
PROTEINS
- (iii) NUMBER OF SEQUENCES: 33
- (iv) CORRESPONDENCE ADDRESS:
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  - (C) CITY: Rochester
  - (D) STATE: New York
  - (E) COUNTRY: USA
  - (F) ZIP: 14603
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version  
#1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/842,322
  - (B) FILING DATE: 24-APR-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Goldman, Michael L.
  - (B) REGISTRATION NUMBER: 30,727
  - (C) REFERENCE/DOCKET NUMBER: 176/60171
- (ix) TELECOMMUNICATION INFORMATION:
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Thr Ser Ser Arg Arg Lys Trp Asn Lys Thr Gly His Ala Val Arg  
1                      5                      10                      15  
Ala Ile Gly Arg Leu Ser Ser Thr Gly Ala  
                    20                      25

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Thr Ser Ser Gly Ser Ser Thr Thr Gly Ala  
1                      5                      10

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Arg Arg Trp Lys Lys Asn Phe Ile Ala Val Ser Ala Ala Asn Arg  
1                      5                      10                      15  
Phe Lys Lys Ile Ser Ser Ser Gly Ala Leu  
                    20                      25

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Arg Arg Lys Trp Gln Lys Thr Gly His Ala Val Arg Ala Ile Gly  
1                      5                      10                      15

Arg Leu Ser Ser  
                    20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: not relevant  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Arg Arg Lys Leu Lys Gly Ala Ile Leu Thr Thr Met Leu Ala Thr  
1                      5                      10                      15

Arg Asn Phe Ser  
                    20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 17 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: not relevant  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Val Arg Asn Ile Lys Ser Met Trp Glu Lys Gly Asn Val Phe Ser  
1                      5                      10                      15

Ser

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: not relevant



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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Arg Arg Lys Leu Lys Ala Ala Val Lys Ala Val Val Ala Ser Ser  
1 5 10 15

Arg Leu Gly Ser  
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Phe Met Asn Asn Trp Glu Val Tyr Lys Leu Leu Ala His Ile Arg Pro  
1 5 10 15

Pro Ala Pro Lys Ser Gly Ser Tyr Thr Val  
20 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Arg Lys Glu Val Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met  
1 5 10 15

Ala Arg Val Phe Ser Val Leu Arg  
20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Arg Arg Leu Ile Asp Ala Tyr Ala Phe Arg Ile Tyr Gly His Trp  
1                      5                      10                      15  
  
Val Lys Lys Gly Gln Gln Gln Asn Arg Gly  
                    20                      25

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Arg Gly Lys Phe Lys Val Ile Cys Leu Thr Val Leu Ala Ser Val Arg  
1                      5                      10                      15  
  
Ile Tyr Tyr Gln Tyr Arg Arg Val Lys Pro Gly  
                    20                      25

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Arg Arg Gly Gln Ile Leu Trp Phe Arg Gly Leu Asn Arg Ile Gln  
1                      5                      10                      15  
  
Thr Gln Ile Lys Val Val Asn Ala Phe Ser Ser Ser  
                    20                      25

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## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Arg Lys His Leu Gln Arg Pro Ile Phe Arg Leu Arg Cys Leu Val  
1                      5                      10                      15  
  
Lys Gln Leu Glu Lys  
                    20

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Thr Glu Lys Met Trp Gln Arg Leu Lys Gly Ile Leu Arg Cys Leu Val  
1                      5                      10                      15  
  
Lys Gln Leu Glu Lys  
                    20

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Arg Arg Ala Ile Gly Phe Lys Lys Leu Ala Glu Ala Val Lys Phe  
1                      5                      10                      15  
  
Ser Ala Lys Leu Met Gly Gln

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20

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile	Lys	Pro	Ala	Lys	Arg	Met	Lys	Phe	Lys	Thr	Val	Cys	Tyr	Leu	Leu
1				5				10						15	
Val	Gln	Leu	Met	His	Cys	Arg	Lys	Met	Phe	Lys	Ala				
			20					25							

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ile	Asp	Leu	Leu	Trp	Lys	Ile	Ala	Arg	Ala	Gly	Ala	Arg	Ser	Ala	Val
1				5				10						15	
Gly	Thr	Glu	Ala												
			20												

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Lys Ala His Lys Ala Ala Thr Lys Ile Gln Ala Ser Phe Arg Gly His

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1	5	10	15
Ile Thr Arg Lys Lys Leu Lys Gly Glu Lys Lys			
20		25	

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Lys Thr Ala Ser Pro Trp Lys Ser Ala Arg Leu Met Val His Thr Val
1 5 10 15
Ala Thr Phe Asn Ser Ile Lys Glu
20

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Lys Lys Lys Lys Lys Arg Phe Ser Phe Lys Lys Ser Phe Lys Leu Ser
1 5 10 15
Gly Phe Ser Phe Lys Lys Ser Lys Lys
20 25

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Lys Lys Lys Lys Lys Phe Ser Phe Lys Lys Pro Phe Lys Leu Ser Gly  
1 5 10 15  
Leu Ser Phe Lys Arg Asn Arg Lys  
20

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Lys Gln Gln Lys Glu Lys Thr Arg Trp Leu Asn Thr Pro Asn Thr Tyr  
1 5 10 15  
Leu Arg Val Asn Val Ala Asp Glu Val Gln Arg Asn Met Gly Ser  
20 25 30

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Asp Gln Val Ala Asn Ser Ala Phe Gln Glu Arg Leu Arg Lys His  
1 5 10 15  
Gly Leu Glu Val Ile  
20

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Tyr His Arg Leu Arg Asp Leu Leu Leu Ile Val Lys Arg Ile Val Glu  
1                      5                      10                      15  
  
Leu Leu Gly Arg Arg  
                    20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gln Gln Leu Ala Thr Leu Ile Gln Lys Thr Tyr Arg Gly Trp Arg Cys  
1                      5                      10                      15  
  
Arg Thr His Tyr Gln Leu Met  
                    20

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Arg Ala Ala Cys Ile Arg Ile Gln Lys Thr Ile Arg Gly Trp Leu Leu  
1                      5                      10                      15  
  
Arg Lys Arg Tyr Leu Cys Met Gln  
                    20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid

Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu  
1 5 10

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu  
1                      5                      10                      15

Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln  
20 25

His Ser Gln Gly Thr Phe Thr Thr Ser Asp Tyr Ser Lys Tyr Leu Asp  
1 5 10 15

Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn Thr  
20 25 30



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- (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg	Asp	Ser
1				5				10						15	
Ala	Arg	Leu	Gln	Arg	Leu	Leu	Gln	Gly	Leu	Val					
			20				25								

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 28 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln
1				5				10						15	
Met	Ala	Val	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	Asn				
			20				25								

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Tyr	Ala	Asp	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ala	Ile	Met	Asn	Lys
1				5				10						15	
Ile	Arg	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala	Gln	Gln	Gln	Lys
			20				25					30			

Ser

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## (2) INFORMATION FOR SEQ ID NO:33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Lys	Leu	Trp	Lys	Lys	Leu	Leu	Lys	Leu	Leu	Lys	Lys	Leu	Leu	Lys	Leu
1				5				10					15		

Gly

**WHAT IS CLAIMED IS:**

1. A method of monitoring the amount of a primary ligand in a cell, said method comprising:

constructing a green fluorescent protein complex, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein;

introducing said green fluorescent protein complex into a cell;

determining a base amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength;

determining amount of fluorescence emission over time at said third wavelength when said cell is excited over time at said first wavelength; and

comparing said amount of fluorescence emission over time to said base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates presence of said primary ligand in said cell and wherein changes in said amount of fluorescence emission over time indicate changes in the amount of said primary ligand in said cell over time.

2. The method of claim 1 further comprising quantitating the amount of fluorescence emission over time to estimate the amount of said primary ligand in said cell over time.

3. The method of claim 1 wherein said primary ligand is calmodulin and said primary ligand-binding peptide is a calmodulin-binding peptide.

4. The method of claim 3 wherein said calmodulin-binding peptide has an amino acid sequence as shown in SEQ ID NO:1.
5. The method of claim 1 wherein the first green fluorescent protein is a blue-shifted green fluorescent protein.
6. The method of claim 5 wherein the blue-shifted green fluorescent protein is excited at a first wavelength maxima of 380 nm and emits fluorescence at a second wavelength maxima of 440 nm.
7. The method of claim 1 wherein the second green fluorescent protein is a red-shifted green fluorescent protein.
8. The method of claim 7 wherein said red-shifted green fluorescent protein is excited at said second wavelength maxima of 495 nm and emits fluorescence at a third wavelength maxima of 505 nm.
9. The method of claim 1 further comprising introducing into the cell a secondary ligand which binds to said primary ligand and which is necessary for binding of said primary ligand to said primary ligand-binding peptide.
10. The method of claim 9 wherein said primary ligand is calmodulin and said secondary ligand is  $\text{Ca}^{2+}$ .
11. The method of claim 1 for further monitoring the amount of a secondary ligand in the cell, where said secondary ligand binds to said primary ligand and is necessary for binding of said primary ligand to said primary ligand-binding peptide, said method further comprising:  
introducing said primary ligand into the cell prior to determining the base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates presence of the secondary ligand in said cell and wherein changes in said amount of fluorescence emission over time indicates changes in the amount of said secondary ligand in said cell over time.
12. The method of claim 11 wherein introducing said primary ligand comprises covalently attaching said primary ligand to one of said first green fluorescent

protein or said second green fluorescent protein in said green fluorescent protein complex.

13. The method of claim 11 wherein said secondary ligand is  $\text{Ca}^{2+}$  and said primary ligand is calmodulin.

14. The method of claim 11 wherein said secondary ligand is a metal ion.

15. The method of claim 14 wherein said metal ion is selected from the group consisting of manganese, nickel, and cadmium.

16. The method of claim 11 wherein said secondary ligand is a molecule.

17. A method of screening a peptide for primary ligand-binding activity, said method comprising:

constructing a green fluorescent protein complex, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein;

introducing said green fluorescent protein complex into a cell in the absence of said primary ligand;

determining a base amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength;

adding said primary ligand to said cell;

determining amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates that said peptide has primary ligand-binding activity.

18. The method of claim 17 wherein said primary ligand is calmodulin.

19. A method of screening a molecule for the ability to bind a primary ligand, said method comprising:

constructing a green fluorescent protein complex, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein;

introducing said green fluorescent protein complex into a cell with said primary ligand;

determining a base amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength;

adding a molecule to said cell;

determining amount of fluorescence emission at said third wavelength when said cell is excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission greater than said base amount of fluorescence emission indicates that said molecule has primary ligand-binding activity.

20. The method of claim 19 wherein said primary ligand is calmodulin.

21. A method of screening a peptide for primary ligand-binding activity, said method comprising:

expressing a green fluorescent protein complex in a cell, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the peptide being covalently attached to the second green fluorescent protein;

purifying the expressed green fluorescent protein complex and placing said purified green fluorescent protein complex in a test vesicle;

determining a base amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength;

adding said primary ligand to said test vesicle;

determining amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission less than said base amount of fluorescence emission indicates that said peptide has primary ligand-binding activity.

22. The method of claim 21 wherein said primary ligand is calmodulin.

23. The method of claim 21 wherein said purifying comprises affinity chromatography.

24. The method of claim 21 wherein said test vesicle is a test tube or test well.

25. A method of screening a molecule for primary ligand-binding activity, said method comprising:

expressing a green fluorescent protein complex in a cell, the green fluorescent protein complex comprising:

a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;

a primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and

a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein;

purifying the expressed green fluorescent protein complex and placing said purified green fluorescent protein complex in a test vesicle;

adding said primary ligand to said test vesicle;

determining a base amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength;

adding a molecule to said test vesicle;

determining amount of fluorescence emission at said third wavelength when contents of said test vesicle are excited at said first wavelength; and

comparing said amount of fluorescence emission to said base amount of fluorescence emission, wherein an amount of fluorescence emission greater than said base amount of fluorescence emission indicates that said molecule has primary ligand-binding activity.

26. The method of claim 25 wherein said primary ligand is calmodulin.

27. The method of claim 25 wherein said purifying comprises affinity chromatography.

28. The method of claim 25 wherein said test vesicle is a test tube or a test well.



29. A green fluorescent protein complex comprising:  
a first green fluorescent protein which is excited at a first wavelength and which emits fluorescence at a second wavelength;  
a primary ligand-binding peptide which reversibly binds a primary ligand, said primary ligand-binding peptide having an amino terminal end and a carboxy terminal end, the amino terminal end of the primary ligand-binding peptide being covalently attached to the first green fluorescent protein; and  
a second green fluorescent protein which is excited at said second wavelength and which emits fluorescence at a third wavelength, the carboxy terminal end of the primary ligand-binding peptide being covalently attached to the second green fluorescent protein,  
wherein when said green fluorescent protein complex is excited at said first wavelength, the amount of fluorescence emission at said third wavelength is reduced when said primary ligand-binding peptide is bound to primary ligand as compared to the amount of fluorescence emission at said third wavelength when said primary ligand-binding peptide is not bound to said primary ligand.
30. The green fluorescent protein complex of claim 29 wherein said primary ligand is calmodulin and said primary ligand-binding peptide is a calmodulin-binding peptide.
31. The green fluorescent protein complex of claim 30 wherein said calmodulin-binding peptide has an amino acid sequence as shown in SEQ ID NO:1.
32. The green fluorescent protein complex of claim 29 wherein said first green fluorescent protein is a blue-shifted green fluorescent protein.
33. The green fluorescent protein complex of claim 32 wherein said blue-shifted green fluorescent protein is excited at a first wavelength maxima of 380 nm and emits fluorescence at a second wavelength maxima of 440 nm.
34. The green fluorescent protein complex of claim 29 wherein said second green fluorescent protein is a red-shifted green fluorescent protein.

35. The green fluorescent protein complex of claim 34 wherein said red-shifted green fluorescent protein is excited at said second wavelength maxima of 495 nm and emits fluorescence at a third wavelength maxima of 505 nm.

36. The green fluorescent protein complex of claim 29 further comprising said primary ligand covalently attached to one of said first green fluorescent protein or said second green fluorescent protein.

37. A cell comprising the green fluorescent protein complex of claim 29.

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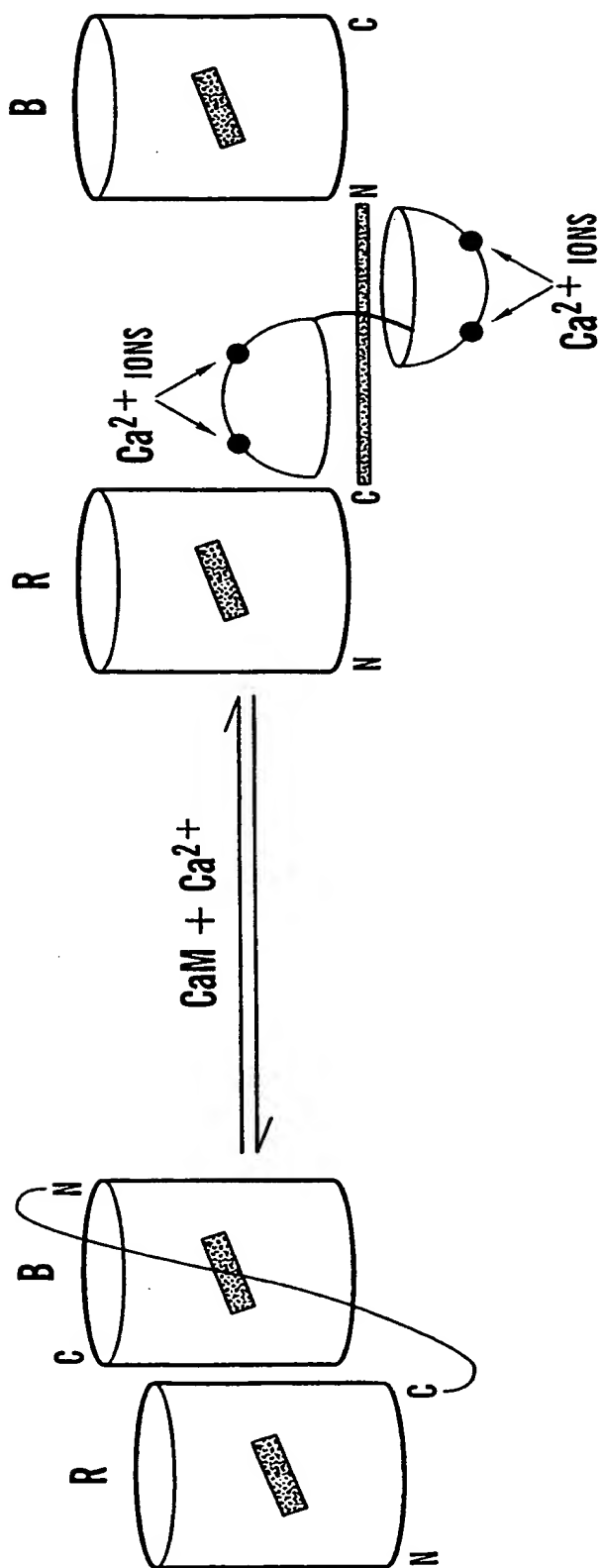
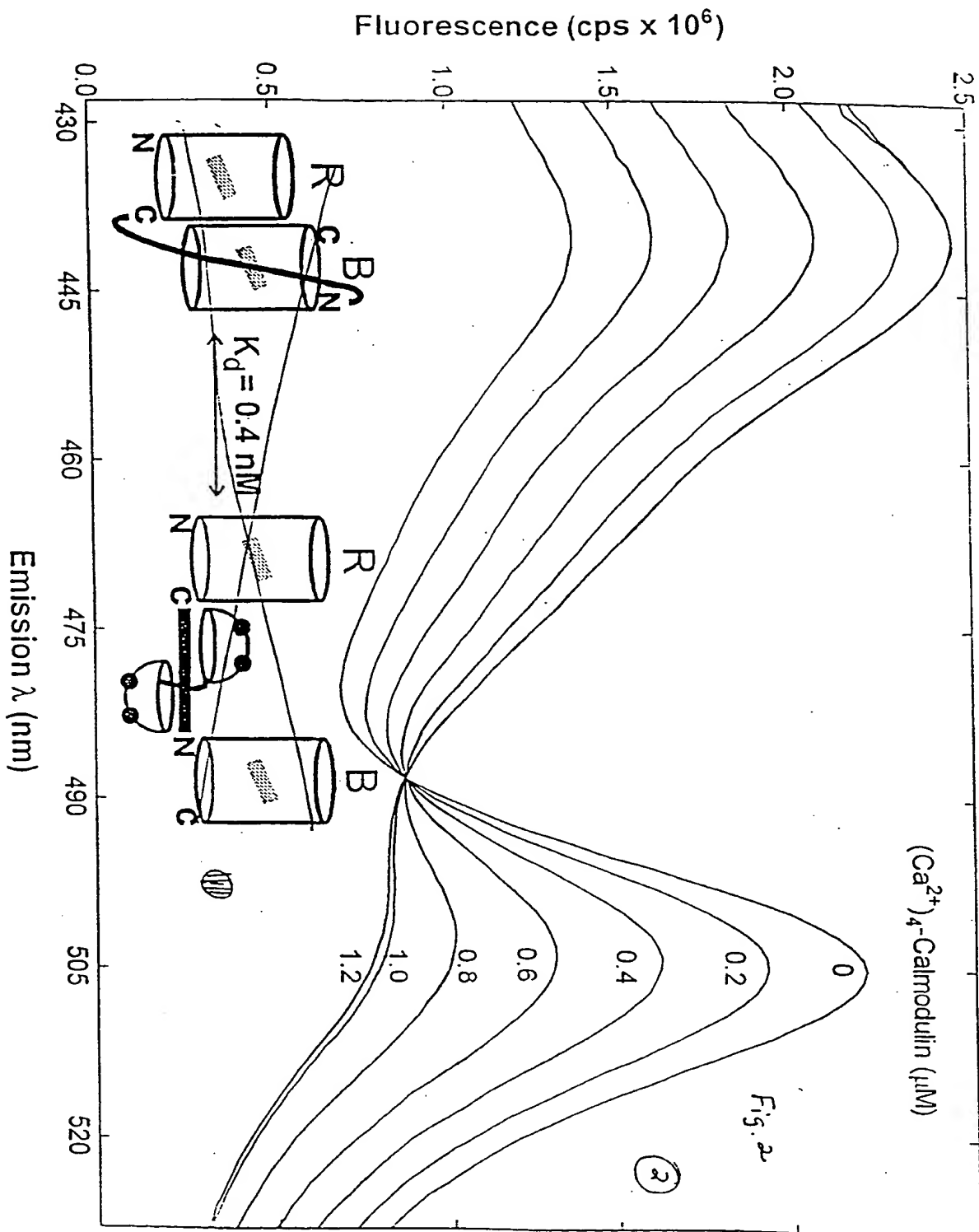
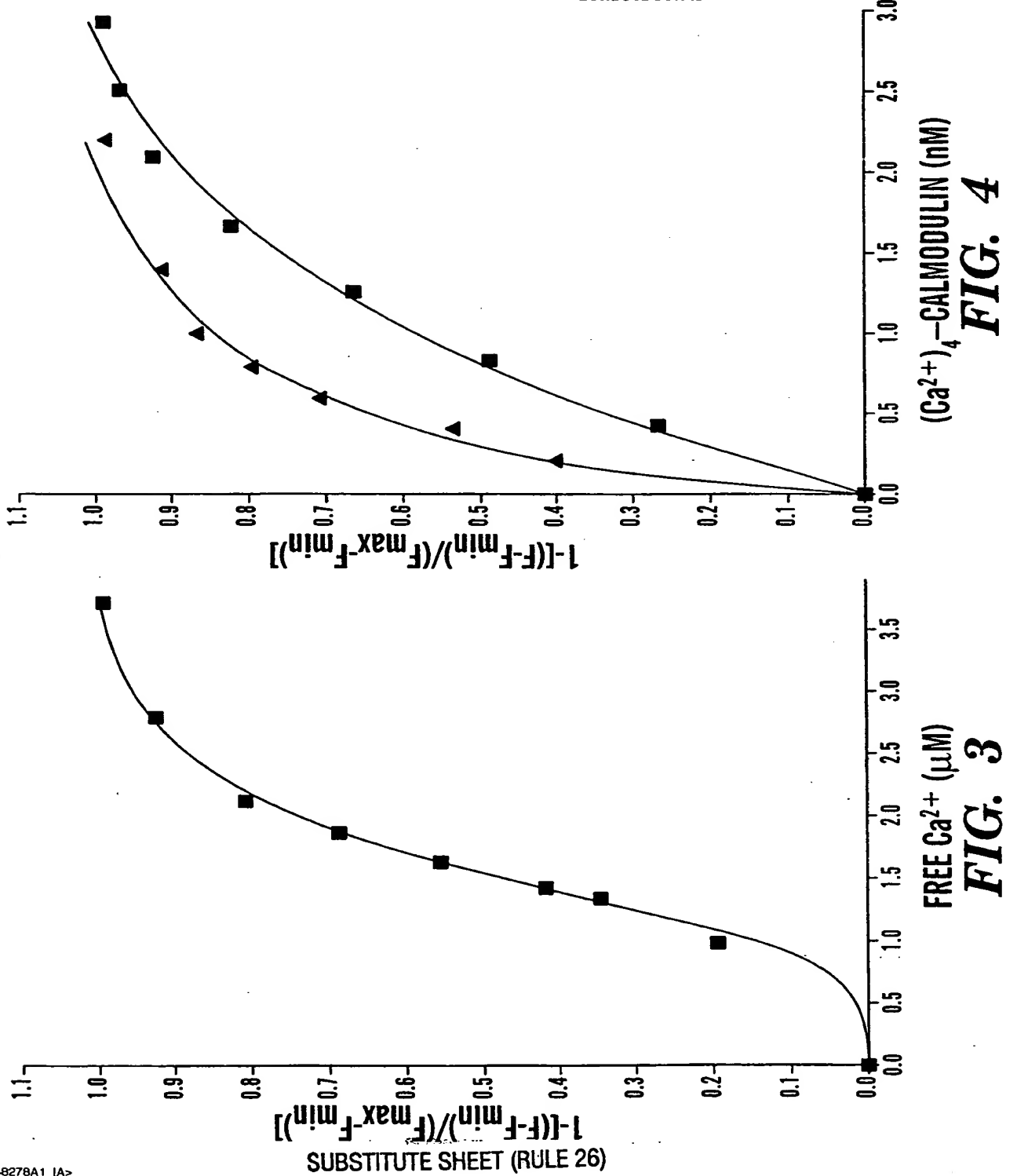


FIG. 1

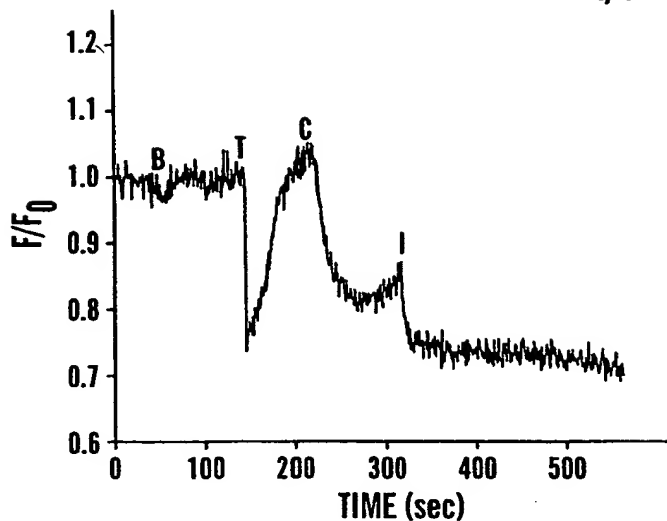
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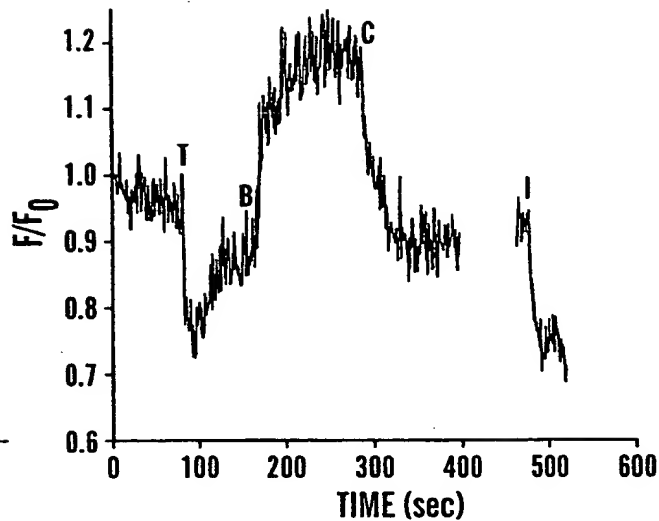
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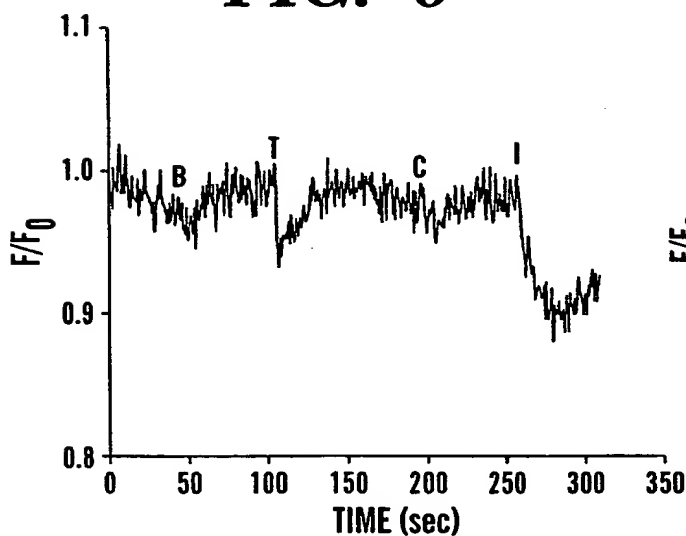
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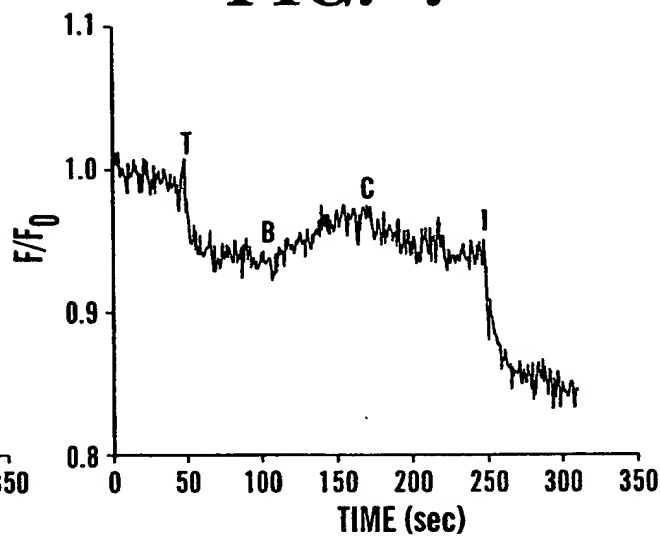
**FIG. 6**



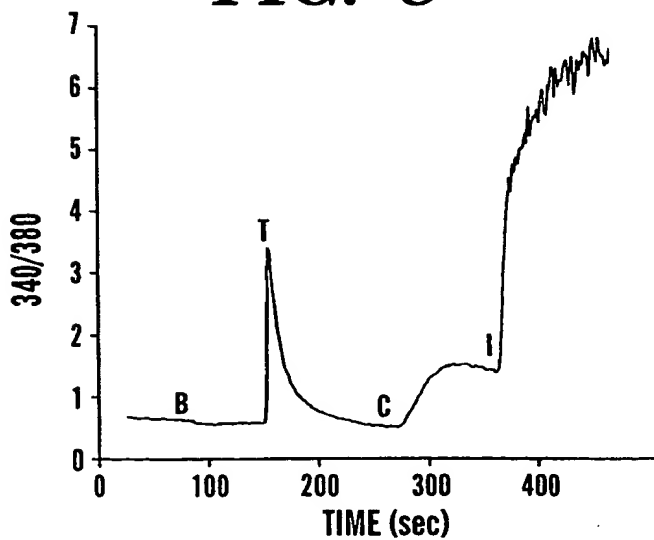
**FIG. 7**



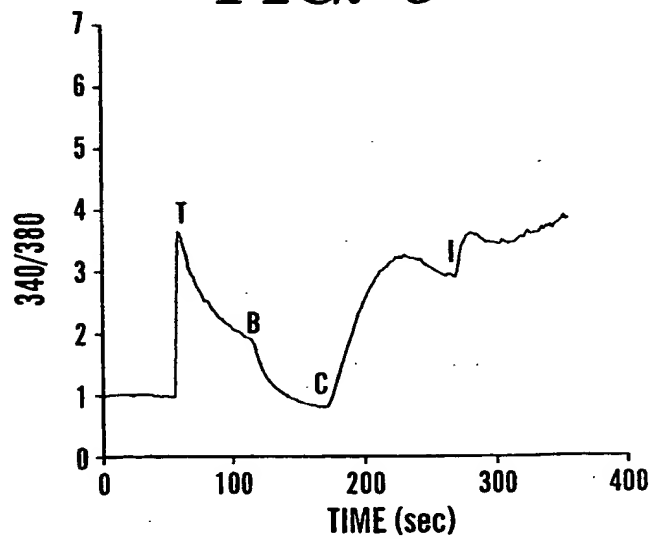
**FIG. 8**



**FIG. 9**



**FIG. 10**



**FIG. 11**

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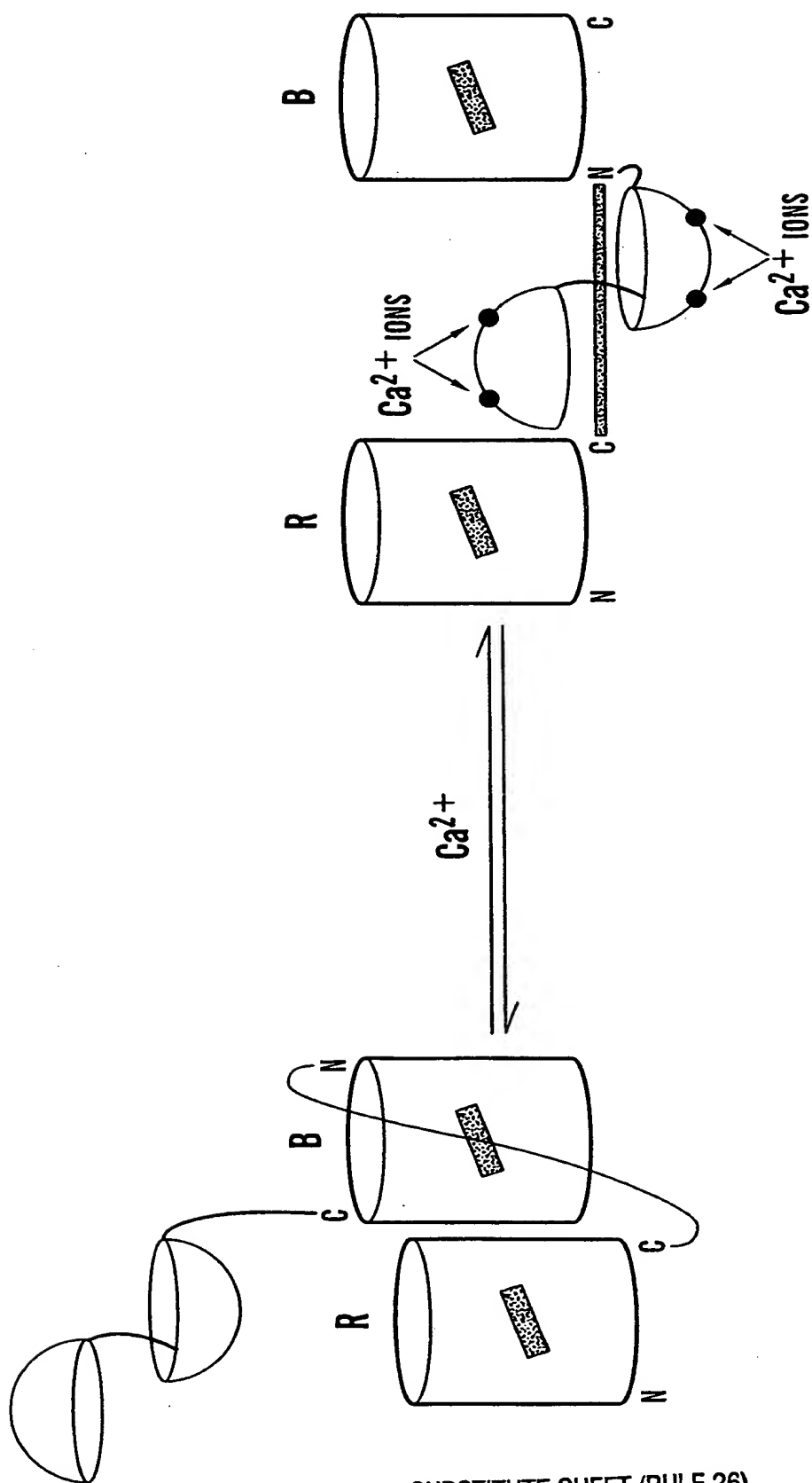


FIG. 12

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FLUORESCENCE CHANGES IN IC-3 (FIP-CA<sub>3</sub>) IN LIVE CELLS

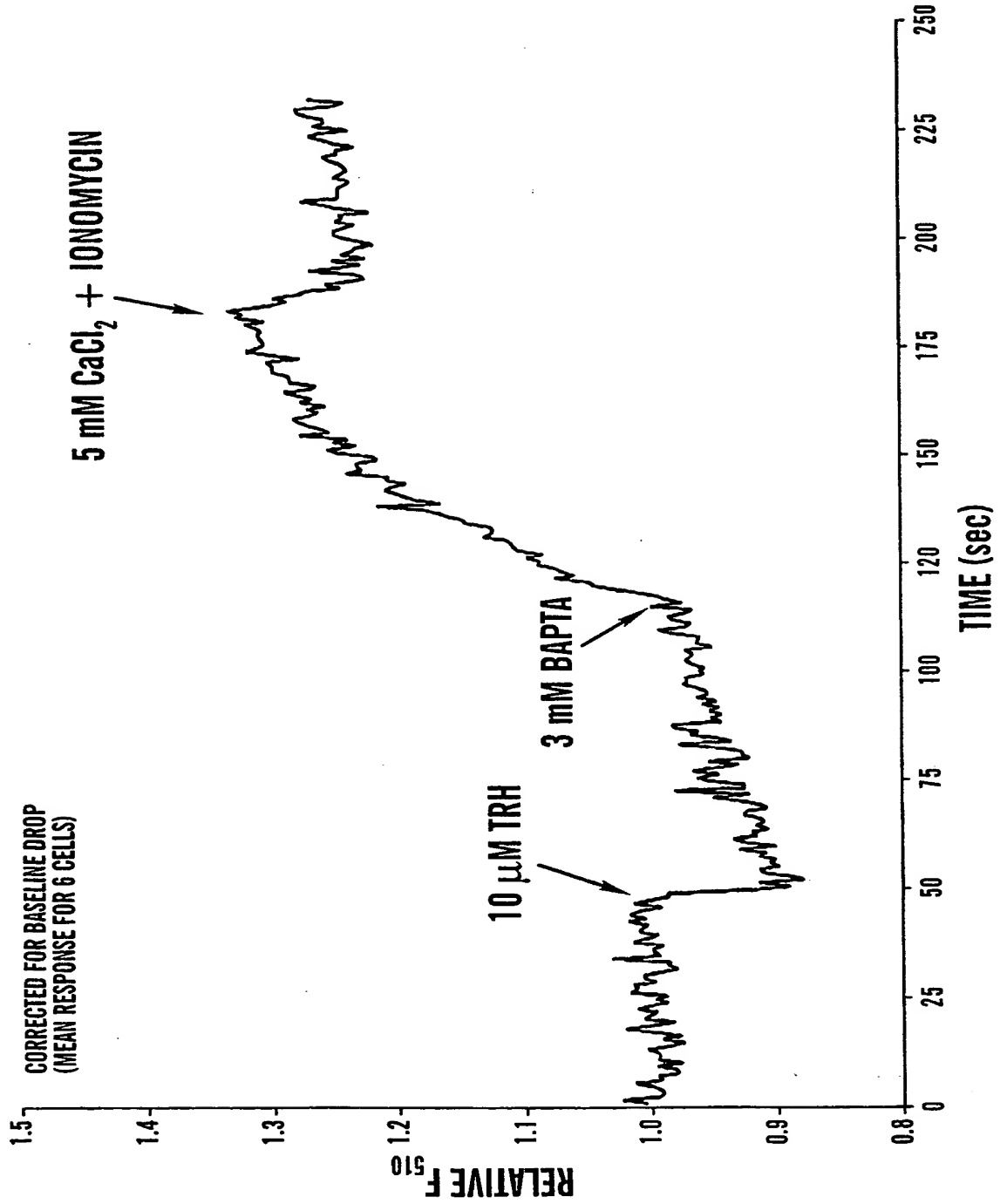


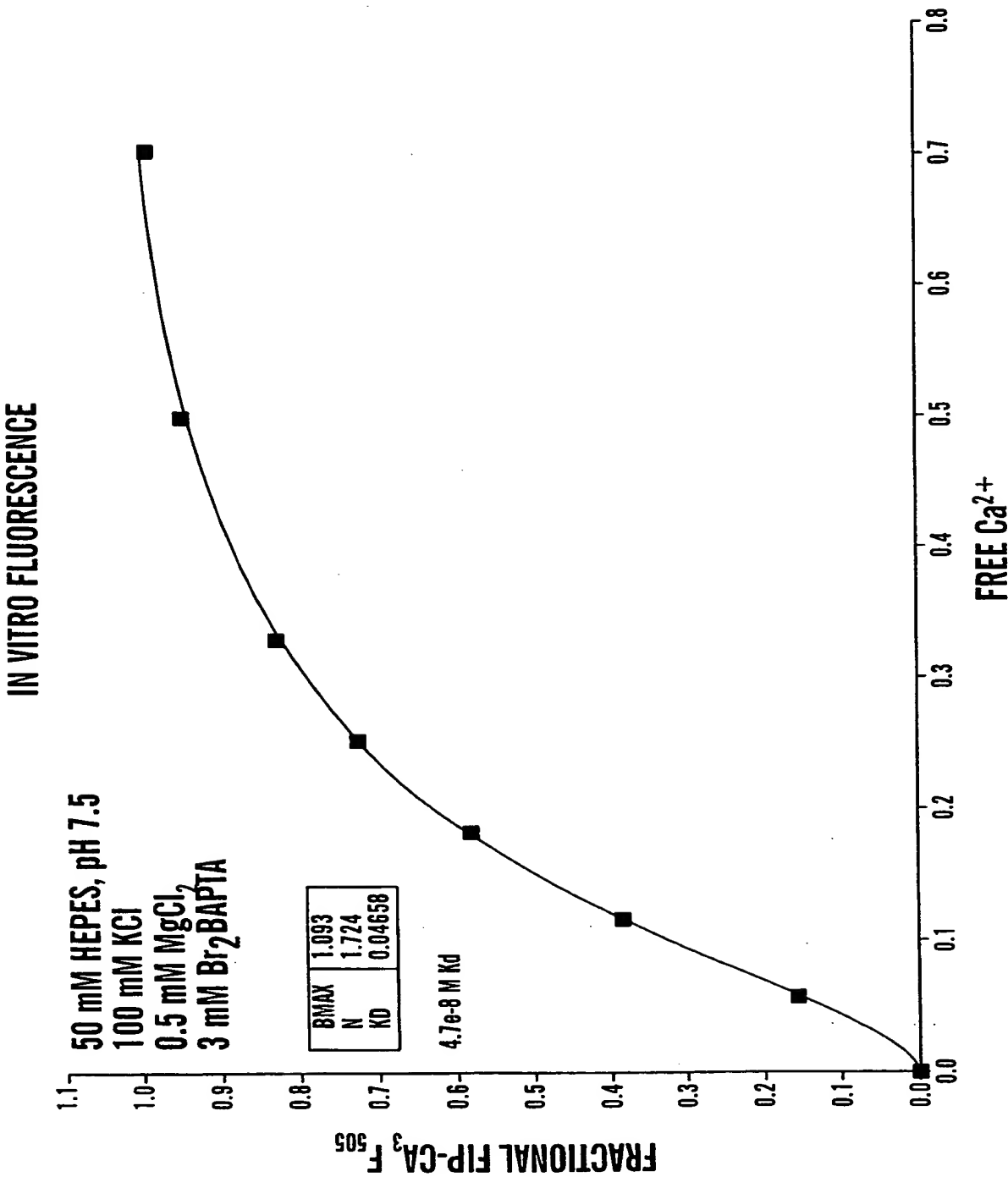
FIG. 13

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FIG. 14



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08109

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/58 G01N33/533 C12N15/65 C07K14/435 G01N33/542

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CHEMICAL ABSTRACTS, vol. 127, no. 5, 4 August 1997 Columbus, Ohio, US; abstract no. 62698, XP002074734 see abstract & V.A. ROMOSER ET AL.: "Detection in living cells of Ca <sup>2+</sup> -dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 20, 16 May 1997, pages 13270-13274, Bethesda MD USA  --- -/--	1-37

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

17 August 1998

Date of mailing of the international search report

27/08/1998

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08109

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHEMICAL ABSTRACTS, vol. 127, no. 23, 8 December 1997 Columbus, Ohio, US; abstract no. 316403, XP002074735 see abstract & A. PERSECHINI ET AL.: "Novel fluorescent indicator proteins for monitoring free intracellular Ca <sup>2+</sup> ." CELL CALCIUM, vol. 22, no. 3, 2 September 1997, pages 209-216, london uk	1-37
A	WO 96 23898 A (NOVO NORDISK A/S) 8 August 1996 see page 2, line 15 - line 16	1-37
A	US 5 491 084 A (M. CHALFIE ET AL.) 13 February 1996 cited in the application see the whole document	1-37

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Information on patent family members

International Application No

PCT/US 98/08109

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